Regulation of RNA interference in the desert locust, *Schistocerca gregaria*

Niels Wynant

Supervisor:
Prof. Dr. Jozef Vanden Broeck

Members of the examination committee:
Prof. Dr. Roger Huybrechts
Prof. Dr. ir. Guy Smagghe
Prof. Dr. Paul Proost
Prof. Dr. Luc Swevers
Dr. Heleen Verlinden
Dr. Gert Simonet

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Niels Wynant

Abstract (Nederlands)

RNA interferentie (RNAi) is een natuurlijk voorkomend verschijnsel dat door dubbelstrengig RNA (dsRNA) geactiveerd wordt en resulteert in het specifiek neerreguleren van genexpressie. RNAi speelt een cruciale endogene rol bij de regulatie van genexpressie, maar is ook uitgegroeid tot een veel gebruikte methode om de functie van genen te achterhalen. Bovendien kan RNAi in de toekomst een potentieel belangrijke rol spelen bij de selectieve bestrijding van plagen veroorzaakt door insectensoorten, enerzijds, en bij de bescherming van zowel nuttige, als vector-insecten tegen virale infecties, anderzijds. Voor deze doeleinden moet het organisme echter een systemische (sys)RNAi-respons vertonen. Dit fenomeen, waarbij injectie of voeden van dsRNA resulteert in een RNAi-response doorheen het lichaam, werd voor het eerst beschreven bij de nematode *Caenorhabditis elegans*. Verschillende andere dieren vertonen echter geen intense sysRNAi. Dit is bijvoorbeeld het geval voor de modelinsecten *Drosophila melanogaster* en *Bombyx mori*, evenals voor alle tot nu toe onderzochte zoogdiersoorten. Enkele recente studies duiden er echter op dat sysRNAi een grote soort variatie vertoont tussen insecten. Deze doctoraatsthesis toont aan dat de injectie van dsRNA in de lichaamsholte van de woestijnsprinkhaan, *Schistocerca gregaria*, resulteert in een zeer gevoelige en robuuste RNAi-respons. Bovendien, kan *S. gregaria* voorgesteld worden als een interessant model voor de studie van de regulatie van de RNAi-gevoeligheid bij insecten. Er werd immers aangetoond dat de efficiëntie van RNAi enigszins varieert tussen verschillende weefsels en dat *S. gregaria* minder gevoelig is voor oraal toegediend dsRNA. In het tweede luik van dit onderzoek werden enkele factoren geïdentificeerd die een rol zouden kunnen spelen bij de weefselafhankelijkheid en het verschil zouden kunnen verklaren in het succes van RNAi bij injectie en oraal toedienen van dsRNA. Aangezien de cellulaire opname van dsRNA een essentiële stap is voor sysRNAi, werd er bovendien een RNAi-transportmechanisme dat werkzaam is bij *S. gregaria* blootgelegd.

De resultaten die in deze doctoraatsthesis gepresenteerd worden, leiden tot nieuwe inzichten in de regulatie van RNAi bij de woestijnsprinkhaan die kunnen bijdragen tot de ontwikkeling van nieuwe methoden of strategieën om de (sys)RNAi-respons te versterken in minder gevoelige insectensoorten.

Abstract

RNA interference (RNAi) is a natural gene silencing mechanism that is triggered by double stranded (ds)RNA. Whereas RNAi is an essential endogenous process of regulating gene expression, it has also become a widely used research tool to knock down and analyse the function of genes in eukaryotes. In addition, several recent studies have shown that RNAi may also contribute to strategies for selectively controlling agricultural pests, including a number of insect species, and might serve to protect beneficial and vector insects against viral infections. Yet, for these purposes, the organism should display systemic (sys)RNAi, a phenomenon that is characterized by the fact that injection or oral delivery of dsRNA induces gene silencing effects throughout the body. SysRNAi-responses were first described in the nematode *Caenorhabditis elegans*, where individual cells have the ability to take up extracellular dsRNA. For other organisms, such as the insect model organisms *Drosophila melanogaster* and *Bombyx mori*, as well as all mammalian species investigated, injection of dsRNA does not result in potent gene-silencing effects. Yet, several recent studies have illustrated that sysRNAi displays a strong inter-species variation in insects. In this PhD-thesis, we demonstrate that the desert locust, *Schistocerca gregaria*, displays a highly sensitive and robust RNAi-response upon injection of dsRNA into its body cavity. Moreover, we present *S. gregaria* as an interesting model to study the regulatory mechanisms of sysRNAi in insects, since the RNAi-efficiency in these locusts varies between different tissues and *S. gregaria* is less sensitive towards orally delivered dsRNA. In a second part of our research, we have identified factors contributing to the tissue-dependency and the distinct RNAi-susceptibility upon injection and oral delivery of dsRNA. Furthermore, regarding the fact that cellular import of dsRNA is a prerequisite for sysRNAi, we identified a functional RNAi-transport route in *S. gregaria*.

Taken together, the data presented in this PhD-thesis significantly improve our knowledge on the regulatory mechanisms of RNAi in the desert locust and might contribute to the development of new methods to enhance the (sys)RNAi-susceptibility in less-responsive insect species.

List of abbrevations

A. aegyptus	*Aedes aegyptus*
A. gambiae	*Anopheles gambiae*
A. mellifera	*Apis mellifera*
A. pisum	*Acyrthosiphon pisum*
ago	*argonaute*
Apo	Apolipophorin
apo1/2	*apolipophorin 1/2 precursor*
B. germanica	*Blatella germanica*
B. mori	*Bombyx mori*
BCA	BiCinchoninic Acid
BSA	Bovine Serum Albumin
C. elegans	*Caenorhabditis elegans*
CHO cells	Chinese Hamster Ovary cells
chs	*chalcone synthase*
ChUP1	Cholesterol Uptake Protein 1
clath	*clathrin heavy chain*
CNS	Central Nervous System
CS	Chondroitin Sulphate
D. melanogaster	*Drosophila melanogaster*
DAG	DiAcylGlycerol
dcr	*dicer*
DS	Dextran Sulphate
dsRBD	double stranded RNA Binding Domain
dsRBP	double stranded RNA Binding Protein
dsRNA	double stranded RNA
EDTA	EthyleneDiamineTetraAcetic
ef1a	*elongation factor 1a*
endoG	*endonuclease G*
eri1	*exoribonuclease 1*

EST	Expressed Sequence Tag
FAO	Food and Agricultural Organization of the United Nations
FHV	Flock House Virus
Fw	Forward
gapdh	*glyceraldehyde phosphate dehydrogenase*
gfp	*green fluorescent protein*
GMS	Gel Mobility Shift
HDL	High Density Lipophorin
IAPV	Israeli Acute Paralysing Virus
L. migratoria	*Locusta migratoria*
LDL	Low Density Lipophorin
loqs	*loquacious*
lpr	*lipophorin receptor*
M. sexta	*Manduca sexta*
mApo	mammalian Apolipoprotein
mHDL	mammalian High Density Lipoprotein
miRNA	micro RNA
mLDL	mammalian Low Density Lipoprotein
mVLDL	mammalian Very Low Density Lipoprotein
p.i.	post injection
piRNA	piwi-interacting RNA
Poly(A)	Polyadenosine
Poly(C)	Polycytosine
Poly(G)	Polyguanidine
Poly(I)	Polyinosine
PVDF	PolyVinyliDene Fluoride
qPCR	quantitative (real time) PCR
RdRP	RNA dependent RNA Polymerase
RISC	RNA Induced Silencing Complex
RNAi	RNA interference

rp49	*ribosomal protein 49*
rsd	*RNA spreading defective*
Rv	Reverse
S. americana	*Schistocerca americana*
S. gregaria	*Schistocerca gregaria*
S. littoralis	*Spodoptera littoralis*
S. litura	*Spodoptera litura*
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
sid	*systemic RNA interference deficient*
siRNA	small interfering RNA
smRNA	small RNA effector molecule
SR	Scavenger Receptor
T. castaneum	*Tribolium castaneum*
TAG	TriAcylGlycerol
TBS	Tris Buffered Saline
tubu	*alpha-tubulin 1a*
ubi	*ubiquitin conjugating enzyme 10*
UTR	UnTranslated Region
vha16	*vacuolar H-ATPase 16*
VSR	Viral encoded Suppressor of RNAi
WCR	Western Corn Rootworm
WSS virus	White Spot Syndrome virus

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Chapter 1

Introduction

Chapter 1: Introduction

1.1. The desert locust

Since many centuries, huge swarms of the desert locust, *Schistocerca gregaria,* pose a threat to agricultural production in the Indian subcontinent, the Middle East and Africa. Desert locusts are grasshoppers belonging to the Acrididae family of the order of the Orthoptera. Locusts have the fascinating ability to occur in different 'phases', a solitarious and a gregarious one (Fig. 1), that can gradually pass into each other and results in obvious differences in behaviour, development, coloration, morphology and physiology.

Fig. 1. Desert locust nymphs in the gregarious (left) and the solitarious (right) phase. Image credit: Tom Fayle, University of Cambridge.

This biological process is termed 'phase transition' and leads to the formation of locust swarms (Chapman and Joern, 1990; Uvarov, 1966). When solitary locusts increase in numbers, due to favourable conditions, they can start to congregate. The locusts will influence each other by tactile, olfactory and visual stimuli, which will elicit a cascade of changes and can eventually cause the solitarious locusts to become gregarious. In the gregarious phase, locusts will be brightly coloured and will actively aggregate in mobile swarms, forming marching bands of juveniles or flying swarms of winged adults. An adult desert locust can consume roughly its own weight in green vegetation and a single swarm can contain billions of animals (Pener, 1991; Uvarov, 1996; Pener and Yerushalmi, 1998; Simpson *et al.*, 2005).

From 2003 until 2005, West Africa faced the largest locust outbreak in 15 years. During this upsurge, more than US$ 400 million were spend to combat locust swarms and harvest losses were estimated at up to US$ 2.5 billion (Food and Agricultural

Organization of the United Nations (FAO)). For many years, the control of locust outbreaks was carried out by spraying millions of litres of chemical pesticides, resulting in toxic non-target effects on humans and environment (Cressman, 1997; van der Valk, 2006). Currently, preventive control and early warning strategies are employed, involving spraying small groups of locusts in minimal quantities before they are able to gather and spread to agricultural areas (FAO).

1.2. Discovery of RNA interference

RNA interference (RNAi), in which double stranded (ds)RNA induces gene silencing, was first reported by Napoli *et al.* (1990) in petunia plants. In an attempt to overexpress *chalcone synthase* (*chs*), a chimeric *chs* gene was introduced that, unexpectedly, resulted in a 50-fold reduction of the levels of the *chs* mRNA. This phenomenon was called *co-suppression of gene expression*, but the molecular mechanisms remained unknown. Several years later, Guo and Kemphues, (1995) used complementary RNA to inhibit expression of the *par-1* gene in the nematode *Caenorhabditis elegans*. To their surprise, expression was also suppressed in the control group that was injected with the sense mRNA strand. Later on, it was found that injection of anti-sense mRNA in *C. elegans* induced a potent silencing signal that could be transferred between different tissues. Moreover, the signal could be passed on to the germ line and maintained for several generations. This prompted the creation of the term RNAi, a mechanism that elicited an active organismal response to foreign RNA and led to the discovery that dsRNA is the effector molecule for RNAi, creating thereby an easy to use method for silencing gene-specific expression (Fire *et al.*, 1998). The Nobel Prize in Physiology or Medicine 2006 was awarded to Andrew Z. Fire and Craig C. Mello for their research on RNAi.

1.3. The RNAi-pathway

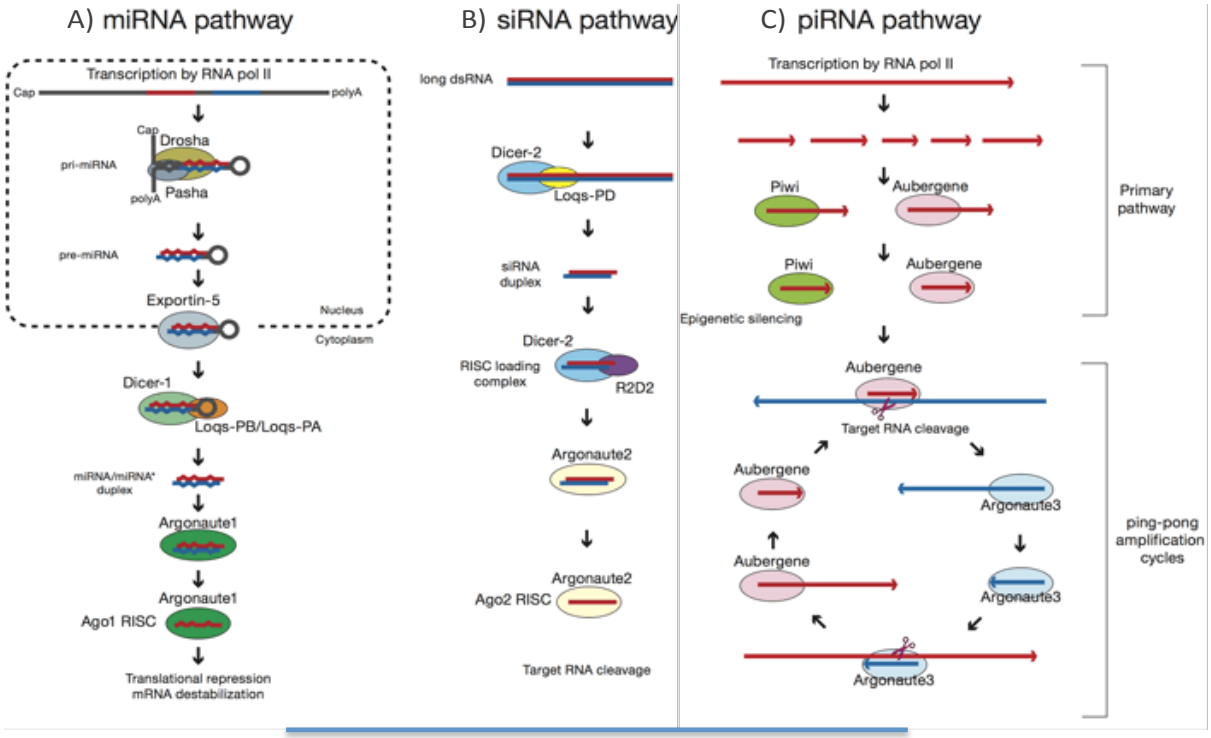
Further elucidation of the RNAi-cascade demonstrated that two ribonuclease machines are at the heart of the mechanism. RNAi is initiated by the RNAse III enzyme Dicer (Dcr) that cleaves long dsRNA into small RNA effector molecules (smRNAs), with a length of approximately 20-25 bp. These smRNAs will in turn be delivered to the RNA induced silencing complex (RISC). RISC will unwind the smRNA

and use the anti-sense strand to locate the complementary mRNA sequence by Watson-Crick base paring. The best-studied outcome is post-transcriptional gene silencing due to cleavage of the mRNA by an RNAseH Argonaute (Ago) enzyme. Besides this more 'classical' RNAi-route, endogenous gene silencing can also be achieved by blocking translation or by transcriptional gene silencing based on heterochromatin remodelling (reviewed in Hammond, 2005).

1.3.1. Small RNA molecules

Based on the structure of the precursor, smRNAs have been classified into three major classes: short interfering (si)RNAs, micro (mi)RNAs and Piwi-interacting (pi)RNAs. The siRNAs act as defenders of the cell in response to intrusion of exogenous nucleic acids and originate from long dsRNA molecules derived from viruses, convergent repeat mobile elements, endogenous inverted repeats or self-annealing transcripts (Sijen and Plasterk, 2003; Wang *et al.*, 2006; Umbach and Cullen, 2009). In contrast, miRNAs are transcribed by the organism's own genome and regulate endogenous gene expression. Mature miRNAs are structurally similar to siRNAs, but have first undergone post-transcriptional modifications. A miRNA is derived from a much longer RNA fragment encoded in the genome that will be trimmed to a stem-loop structure by an RNAse III enzyme called Drosha. This structure will be transported to the cytoplasm where Dcr will cleave it to a mature miRNA molecule that can be integrated into the RISC. In animals, the miRNAs differ from siRNAs in that miRNAs have incomplete base pairing to a target and regulate the transcript levels of many different mRNAs with similar sequences. Typically, siRNA-directed RNAi results in degradation of the mRNA, while miRNA-directed gene silencing is established by translational inhibition (Carthew and Sontheimer, 2009). The piRNAs, which are a bit longer (24-31 nt long), differ from siRNAs and miRNAs in several aspects and silence transposons and retrotransposons. The piRNA sequences are extremely diverse, with over 1.5 million distinct piRNA identified in flies thus far. All these piRNA genes are clustered in several hundred genomic loci. In contrast to miRNA- and siRNA-directed RNAi, piRNA-directed silencing is independent of Dcr enzymes (Saito *et al.*, 2006; Vagin *et al.*, 2006). Although, the piRNA-pathway is not well understood, the presence of a primary processing pathway is suggested, where the piRNAs are transcribed from a piRNA-precursor. In addition, in flies a ping-pong

amplification mechanism is described, wherein primary piRNAs bind to their complementary targets, cause the recruitment of Piwi proteins and generate secondary piRNAs from the transcript target (Brennecke *et al.*, 2007) (Fig. 2).



Drosophila

Fig. 2. The miRNA-, siRNA- and piRNA-pathway in *Drosophila* (Diptera). (A) The miRNA-biogenesis starts with the synthesis of a pri-miRNA that will be processed by Drosha (in green) and the dsRNA binding cofactor Pasha (in grey) into a pre-miRNA. After translocation to the cytoplasm, Dicer-1 (green) and Loquacious (loqs, orange) will produce mature miRNA duplexes. An Argonaute1 (green) containing protein-complex, termed RISC, will subsequently use the mature miRNA to inhibit the translation of complementary sequences in the cell. (B) Long dsRNA-fragments, derived from viruses or transposons, activate the siRNA-pathway. Dicer-2 (blue), with the help of the dsRNA binding cofactor R2D2 (purple), will process the dsRNA into siRNAs. Next, an argonaute2-containing RISC will use the siRNAs to locate complementary transcript sequences in the cell, which will be cleaved by Argonaute2. (C) Primary piRNAs will be transcribed by the RNA polymerase II and bound by the PIWI proteins Piwi and Aubergene that can directly target transcripts or generate epigenetic silencing. In addition, secondary piRNAs can be produced by means of a ping-pong amplification cycle, where the Aubergene will cleave the target transcripts into secondary piRNAs. By the action of Argonaute3, sense targets can be targeted. Adapted image from: Institute of Molecular Biology, University of Oregon.

1.3.2. Dicer

Although diverse proteins are involved in the production of the smRNAs, the processing and effector steps of the RNAi-response are mediated in a common mode in fungi, plants, worms, insects and vertebrates, with a central role for the core components Dcr and Ago (Ghildiyal and Zamore, 2009). Several domains in a specific order from the carboxy terminus typically characterize Dcr enzymes: a DEXD/H helicase domain, a DUF 283 domain, a PAZ domain, two tandem RNase III domains and a dsRNA-binding domain (dsRBD). Some members of the family differ from this general arrangement, for instance some lack a functional helicase domain or PAZ-domain, or the number of dsRBD can range from zero to two (Carthew and Sontheimer, 2009). PAZ domains are specialized in binding ends of RNA molecules. Each of the two RNAse III domains then cleaves one of the two RNA strands, leading to duplex scission with a new 3' overhang of 2 nt. In flies and worms, the helicase domain promotes dsRNA processing, while the function of the DUF283 remains largely unknown (Fig. 3) (Zhang *et al.*, 2004).

The number of Dcr enzymes can differ between different organisms. Where yeast, nematodes and vertebrates have only one Dcr protein, insects have two Dcr proteins (Dcr1 and Dcr2), which produce miRNAs and siRNAs, respectively (Lee *et al.*, 2004). RNase III enzymes often need dsRNA binding protein (dsRBP). The *Drosophila* Drosha enzyme binds to the dsRBP Pasha and Dcr-1 binds to Loquacious (Loqs) to generate miRNA duplexes (Fig. 2A). Another dsRBP, R2D2, binds to Dcr-2 and is required to load siRNAs into the RISC (Fig. 2B) (Ghildiyal and Zamore, 2009).

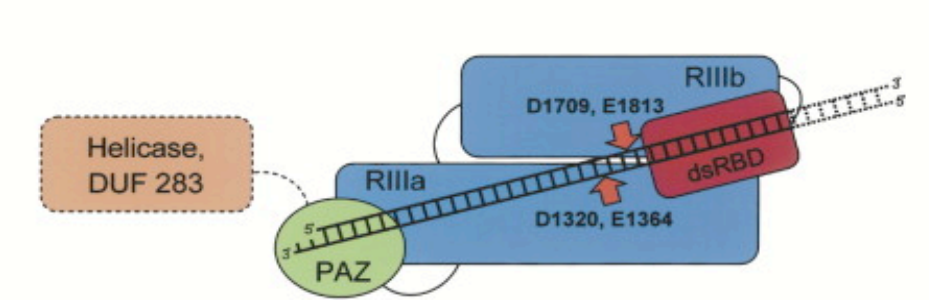


Fig. 3. A model for dsRNA processing by Dicer. The exact mode of action of the Helicase and DUF 283 domain remains largely undetermined. These domains are therefore delineated with broken lines. The RNase III domains (RIIIa and RIIIb, blue) cleave the RNA strands (indicated with the orange arrows), while the dsRNA binding domain (dsRBD, red) assists the dsRNA-binding and the PAZ-domain (green) binds to the 3' end of the dsRNA. Image credits: Zhang *et al.* (2004).

1.3.3. Argonaute

Ago proteins are defined by the presence of five domains: a PAZ domain (shared with Dcr enzymes), a PIWI domain (specific to the Argonaute superfamily), a domain of unknown function (DUF 1785), a N domain and a Mid domain (Carthew and Sontheimer, 2009). Crystallography and structural studies with different Ago proteins have illuminated many of its functions (Song *et al.*, 2004; Wang *et al.*, 2008a; Wang *et al.*, 2008b; Frank *et al.*, 2010). The PAZ domain has a 3' end RNA binding activity, a function that will be used to bind the siRNA guide strand. The other (5') end of the strand will display phosphate binding in a pocket with the Mid-domain. The remaining of the strand will interact with a positively charged surface, to which all domains contribute, stacking the guide strand nucleotides 2-6, which are especially important for target recognition. Their Watson-Crick side faces towards the exterior, making them available for base pairing. The PIWI domain adopts an RNase H like fold that, in some cases, can catalyse endonuclease activity of the mRNA target (Fig. 4). Yet, not all Ago proteins have endonuclease activity.

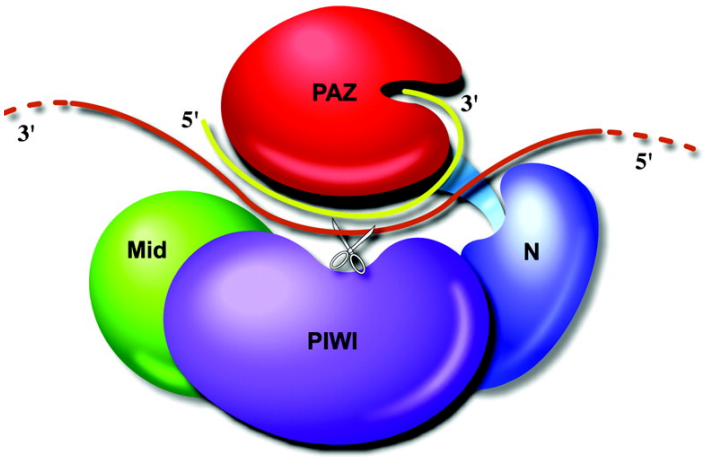


Fig. 4. Schematic depiction of the model for siRNA-guided mRNA cleavage by Argonaute. The PAZ cleft will bind the 3' end of the siRNA (yellow). The mRNA (red) will be stacked between the N- (blue) and PAZ-domain (red) and between the Mid- (green) and PAZ-domain. The active site of the PIWI domain (purple) cleaves (illustrated with scissors) the mRNA opposite the middle of the siRNA guide strand. Image credits: Song *et al.* (2004).

The Argonaute superfamily is segregated into two clades, the Ago and the Piwi clade. In the genome of all animals investigated so far both clades are found, whereas in plants and fungi only the Ago clade is present (Cerutti and Casas-Mollano, 2006). In *Drosophila*, there are three Piwi members: Piwi, Aubergene and AGO3. They are expressed in the male and female germ line cells. Piwi is also expressed in somatic cells that are in close contact with germline cells. Moreover, animals mutant in the Piwi-proteins have defects in germ cell development. Furthermore, the *Drosophila* Piwi was shown to be needed to maintain germ line cells in both testes and ovaries, and use piRNAs as a guide to control mobile genetic elements (Cox *et al.*, 1998; Cox *et al.*, 2000; Megosh *et al.*, 2006).

Most species contain multiple *ago* genes. For example, five, eight, and seventeen paralogs are present in *Drosophila*, humans and *C. elegans*, respectively (Carthew and Sontheimer, 2009). Functional specialization between siRNA- and miRNA-silencing is very clear in flies and worms, while in humans four of the eight Ago proteins are from the Ago clade and associate with both siRNAs and miRNAs (Tomari and Zamore, 2005). In flies, Ago2 mainly mediates siRNA-directed mRNA cleavage and Ago1 is mostly involved in miRNA-directed translational inhibition (Okamura *et al.*, 2004; Siomi *et al.*, 2004; Forstemann *et al.*, 2007). In addition, worms have the ability to amplify cellular dsRNA-levels (described in detail in § 1.4.3). For this purpose, they possess primary and secondary Agos that work sequentially to direct gene silencing (Yigit *et al.,* 2006).

1.4. RNAi as a tool

Whereas RNAi has a crucial role in regulating cellular gene expression and immunity against genomic parasites, it can also be exploited as a powerful research tool for reverse genetic studies. This has prompted the development of large-scale RNAi-based functional genomic screens in *C. elegans*, *D. melanogaster* and human cell lines (Fraser *et al.*, 2000; Berns *et al.*, 2004; Boutros *et al.*, 2004; Paddison *et al.*, 2004). RNAi has also revolutionized functional biology in non-model organisms and species that are not feasible to be genetically transformed. In addition, RNAi has many other application possibilities, including medical treatment for viral infection and diseases, agricultural manipulation and pest management.

It may not come as a surprise that soon after the discovery of RNAi, there was a lot of interest in utilizing RNAi in pharmaceutics. Yet, using RNAi in mammals was initially challenging, since introduction of long dsRNA molecules into mammalian cells initiates the interferon pathway, an innate immune response that causes a general inhibition of translation, abrogating the specificity of RNAi. However, later on it was demonstrated that siRNAs do not activate the interferon pathway, but trigger RNAi in mammalian cells (Elbashir *et al.*, 2001). This heralded the search for RNAi-based therapy. Over the past decennia, an excess of *in vitro* and *in vivo* proof of concept studies have shown that practically every human disease with a gain of function genetic disorder can become a target for therapeutic RNAi (Bumcrot *et al.*, 2006; de Fougerolles *et al.*, 2007; Kim and Rossi, 2007). (Pre-)clinical trials have been successful in improving a wide range of disease associated phenotypes including neurodegenerative diseases (Davidson and Boudreau, 2007), metabolic disorders (de Fougerolles *et al.*, 2007), ocular maladies (Campochiaro, 2006) and cancer (Gartel and Kandel, 2006; Chen *et al.*, 2007). Moreover, the RNAi-technology has been successfully used to suppress replication of different viruses, including human immunodeficiency virus (HIV), dengue virus, hepatitis B, hepatitis C and poliovirus (Haasnoot *et al.*, 2003; Leonard and Schaffer, 2006).

In the field of biotechnology, RNAi has been used to engineer plants with increased nutritional values. For example, maize plants have been produced with higher levels of the essential amino acid lysine (Segal *et al.*, 2003) and cotton plants with increased fatty acid content in their seed oil (Liu *et al.*, 2002). RNAi-based genetic engineering has also been used to reduce the levels of undesirable products, for instance cotton stocks with reduced toxins in their seed (Sunilkumar *et al.*, 2006), tomatoes that contain lower levels of allergens (Le *et al.*, 2006), and precursors of likely carcinogens have been reduced in tobacco plants (Gavilano *et al.*, 2006). Finally, RNAi has great potential to contribute to the development of novel strategies for selectively controlling agricultural pests. *In planta* expression or spraying of dsRNA that is directed against suitable eukaryotic pest target genes has been shown to give crops resistance towards pest organisms, including insects (Baum *et al.*, 2007; Mao *et al.*, 2007; Huvenne and Smagghe, 2010), nematodes (Steeves *et al.*, 2006; Huang *et al.*, 2006) and parasitic weeds (Aly *et al.*, 2009; Niu *et al.*, 2010). Finally, RNAi-based

transgenic plants with increased resistance against multiple viral pathogens have also been successfully developed (Duan *et al.*, 2012).

1.5. Systemic RNA interference

In many plants, fungi and invertebrates, RNAi can be obtained by injection of 'naked' dsRNA into the circulatory system. The process in which RNAi is triggered distant from the site of dsRNA-administration is generally termed systemic (sys)RNAi and was first demonstrated in *C. elegans*, where an RNAi-response could be observed throughout the body, regardless of the site of dsRNA-injection (Fire *et al.*, 1998). Later on, it was noted that a sysRNAi-response could also be induced upon feeding them with bacteria that produce dsRNA-molecules and even upon soaking the worms in a solution that contains dsRNA (Timmons and Fire, 1998; Timmons *et al.*, 2001). Furthermore, in addition to the ability to take up dsRNA from the environment, nematode cells further transport the RNAi-signal to neighbouring cells and tissues (Winston *et al.*, 2002; Jose *et al.*, 2009).

1.5.1. Transmembrane channel mediated uptake of dsRNA

The best-studied mechanism of cellular uptake of dsRNA in animals is the transmembrane channel mediated uptake mechanism by SID1 that is necessary for the import of dsRNA into *C. elegans* cells. The *systemic RNA interference deficient 1* (*sid1*) mutant was identified in a forward genetic screen conducted by Winston *et al.* (2002). Expression of the *Ce*-SID1 in *D. melanogaster* S2 cells, which lack a *sid1* homologous sequence, was sufficient to enhance the dsRNA-uptake from the medium, apparently by a passive mechanism (Feinberg and Hunter, 2003). Furthermore, it was shown that SID1 mediates the import but not the export of dsRNA (Jose *et al.*, 2009).
Homologous sequences of *sid1* in fish and mammals were also identified to mediate dsRNA-import (Duxbury *et al.*, 2005; Ren *et al.*, 2011; Elhassan *et al.*, 2012). The genomes of most insects, with the exception of dipterans, possess genes related to *sid1*. Some insect species have several copies, for example, there are three paralogs of *sid1* in the red flour beetle, *Tribolium castaneum* (Coleoptera), and in the silk moth, *Bombyx mori* (Lepidoptera). Yet, silencing of the three *T. castaneum sid1 like* sequences, either individually or simultaneously, had no effect on the sysRNAi-

response (Tomoyasu *et al.*, 2008). Moreover, a recent publication suggested that the highly robust sysRNA-response in the migratory locust, *Locusta migratoria* (Orthoptera), does not require SID1 (Luo *et al.*, 2012). On the other hand, a study has shown that the *sid1* levels in the honey bee, *Apis mellifer*a (Hymenoptera), were up-regulated after administering dsRNA, suggesting thereby a role in the RNAi-response (Aronstein *et al.*, 2006). At present, it remains an open question whether *sid1 like* genes are required for the spreading of the RNAi-signal in insects.

1.5.2. Endocytosis-based uptake

1.5.2.1. The endocytosis mechanism

Eukaryotic cells can take up particles from the extracellular medium by surrounding an area with the plasma membrane, which then buds off inside the cell to form a vesicle containing the engulfed material. Endocytosis is used for the ingestion of large particles (such as bacteria) and the uptake of fluids and macromolecules in small vesicles. The former is known as phagocytosis, while the latter can be further subdivided into pinocytosis and receptor-mediated endocytosis. Pinocytosis is used primarily for the absorption of extracellular fluids. Unlike receptor-mediated endocytosis, pinocytosis is non-specific in the substances that are transported. Macromolecules to be internalized by receptor-mediated endocytosis first bind to specific cell surface receptors. Typically, these receptors are concentrated in specialized regions on the plasma membrane, termed clathrin-coated pits. These pits will be internalized to form clathrin-coated vesicles that contain both receptor and ligand. Subsequently, these vesicles will fuse with early endosomes, where they will be recycled to the plasma membrane, delivered to the cytoplasm or transported to the lysosomes for degradation. An important feature of early endosomes is that they maintain an acidic internal pH by the action of vacuolar H-ATPases. The acidic pH leads to the dissociation of the ligand and receptor complex and can activate endosomal transporters (Summarized in Huotari and Helenius, 2011). Recent studies have demonstrated that cells also possess clathrin-independent receptor mediated endocytosis uptake routes. In particular, the protein caveolin-1 plays an essential role in the forming of small invaginations in the plasma membrane, which are called caveolae. Caveolar endocytosis is believed to play role in cell-signalling,

mechanosensing and it has been shown that it can serve as a cell entry route for particular pathogens. The pathogens exploiting this endocytotic pathway include viruses such as the polyoma and SV40 virus and bacteria such as some strains of *Porphyromonas gingivalis, Pseudomonas aeruginosa* and *Escherichia coli* (Reviewed in Parton and Simons, 2007).

1.5.2.2. Endocytosis-based dsRNA-transport

In *C. elegans*, dsRNA is taken up from the gut lumen via endocytosis, in a SID2-dependent manner (McEwan *et al.*, 2012). Worms that are mutant for *sid2* are refractory towards orally delivered dsRNA but display RNAi when the dsRNA is injected into the pseudocoel (Winston *et al.*, 2007). In addition, expression of *Ce*-SID2 in S2-cells severely enhanced the dsRNA-uptake. Nevertheless, *sid1* mutant worms fed with dsRNA fail to show silencing, including in the midgut cells (Winston *et al.*, 2007; McEwan *et al.*, 2012; Zhuang and Hunter, 2012). Thus, the dsRNA might be taken up via SID2-mediated endocytosis, but delivery to the cytoplasm seems to require SID1. It has also been suggested that SID-1 locates on the endosomes of *C. elegans*, suggesting a role in the export of dsRNA from the endosome to the cytoplasm (McEwan *et al.*, 2012). Interestingly, whereas the related nematode *C. briggsae* is competent in sysRNAi upon injection, but not upon feeding dsRNA, expression of the *Ce*-SID-2 transporter in the gut of *C. briggsae* render these worms sensitive to feeding of dsRNA (Winston *et al.*, 2007).

A second forward genetic screen identified three RNA spreading defective (*rsd*) mutant worms (*rsd-2*, *rsd-3* and *rsd-6*) that remained sensitive to sysRNAi in somatic cells but were resistant to a response in germ line cells. Whereas, the function of *rsd-2* and *rsd-6* remains largely unknown, *rsd-3* encodes a homolog of the human Enthoprotin that is required for clathrin-dependent membrane budding during the internalization step of receptor-mediated endocytosis (Tijsterman *et al.*, 2004). Moreover, it has been shown that SID-3, which is the nematode homolog of the activated cdc-42-associated kinase (ACK), promotes dsRNA-uptake in *C. elegans*, presumably by promoting endocytosis (Jose *et al.*, 2012). In addition, silencing of genes that are associated with the endocytosis pathway could reduce the sysRNAi-sensitivity in *C. elegans* (Saleh *et al.* 2006). Yet, this shouldn't necessarily imply a role for endocytosis in the uptake of dsRNA, since the endosome-associated protein SID-5

was identified to exert export of the dsRNA and mediate the intercellular dsRNA-transport between cells (Hinas *et al.*, 2012). The action of the different SID-proteins in the transport of dsRNA in *C. elegans* is summarized in Fig. 5.

D. melanogaster displays no robust sysRNAi-response and lacks *sid1 like* sequences. Still, *Drosophila* S2 cells can take up dsRNA from their medium, suggesting the presence of an alternative (*sid-1* independent) dsRNA-uptake mechanism. Two independent functional screens, Saleh *et al.* (2006) and Ulvila *et al.* (2006), identified receptor-mediated endocytosis as a key player in the dsRNA-uptake by S2 cells. Furthermore, it was shown that scavenger receptors (SRs) mediate clathrin-dependent endocytosis of the dsRNA (Saleh *et al.*, 2006; Ulvila *et al.*, 2006). SRs constitute a group of structurally unrelated pattern recognition receptors that endocytose anionic macromolecules and (modified) lipoproteins. In general, SRs have been referred to the recognition of foreign body particles and signals of the innate immunity. In mammals, this family of receptors are categorized in, at least, six classes according to their structural characteristics (classes A-F) (Gough and Gordon, 2000; Areschoug and Gordon, 2009). *D. melanogaster* has two classes of SRs, B and C. Surprisingly, the class C SRs are the functional homologous of the mammalian class A SRs (Ramet *et al.*, 2001). In addition, another *Drosophila* SR, termed Eater, has been identified to recognize Gram-positive and –negative bacteria (Erturk-Hasdemir and Silverman, 2005; Kocks *et al.*, 2005). Silencing *SR-CI* and *eater* led to a significant decrease in the internalization of dsRNA fragments, while silencing the class B SRs *crq*, *emp* and *ninaD* had no detectable effect on the uptake of dsRNA (Ulvila *et al.* (2006).

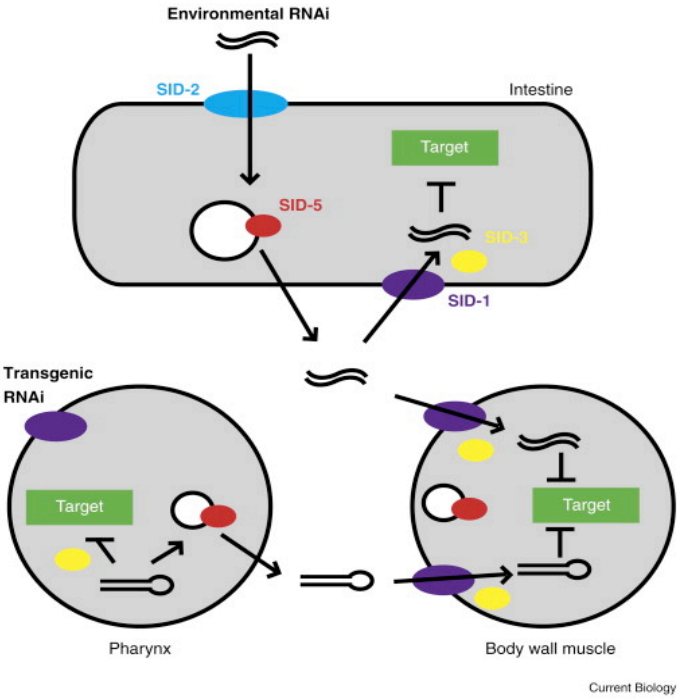


Fig. 5. The action of the different SID-proteins in the transport of dsRNA in *C. elegans*. Ingested dsRNA (double black line) enters the body via SID-2 (blue). SID-5 (red) is associated with endosomes and is required for the export of dsRNA into the pseudocoelom. SID-1 (purple) and SID-3 (yellow) are required to import dsRNA back into the tissues. Similarly, during transgenic RNAi, SID-5 is required for the export of dsRNA, which will be taken up by distant tissues via SID1 and SID-3. It must however be noted that SID-1 might also be present on the endosomes, redirecting the dsRNA from the normal endocytotic uptake route to the cytoplasm (this possible alternative function for SID-1 is not included in this figure). Image credit: (Rocheleau, 2012).

1.5.3. Amplification of the RNAi-signal

The RNAi-response can be further potentiated through the action of an RNA-dependent RNA-polymerase (RdRP) system that converts small populations of exogenously-encountered dsRNA fragments into an abundant pool of siRNAs. The amplification of the siRNA-pool is initiated when primary siRNAs bind to their mRNA targets and direct cleavage by Ago. In plants, RdRPs use these cleaved transcript fragments as template to synthesize long dsRNA, which is subsequently diced into secondary siRNAs (Fig. 6A). On the other hand, in *C. elegans*, binding of a primary Ago, termed RDE-1, to the mRNA-target will recruit RdRPs to synthesize secondary siRNAs (Fig. 6B) (Ghildiyal and Zamore, 2009). *RdRps* are not as widely distributed among eukaryotes as *dcr*- and *ago*-genes, with *RdRP-like* sequences absent in the genomes of

vertebrates and insects. Nevertheless, a monophyletic origin for the *RdRPs* in nematodes, fungi and plants is supported (Cerutti and Casas-Mollano, 2006). Interestingly, *RdRPs-like* sequences have been found in several (non-insect) arthropods, such as in ticks and mites (Kurscheid et al., 2009).

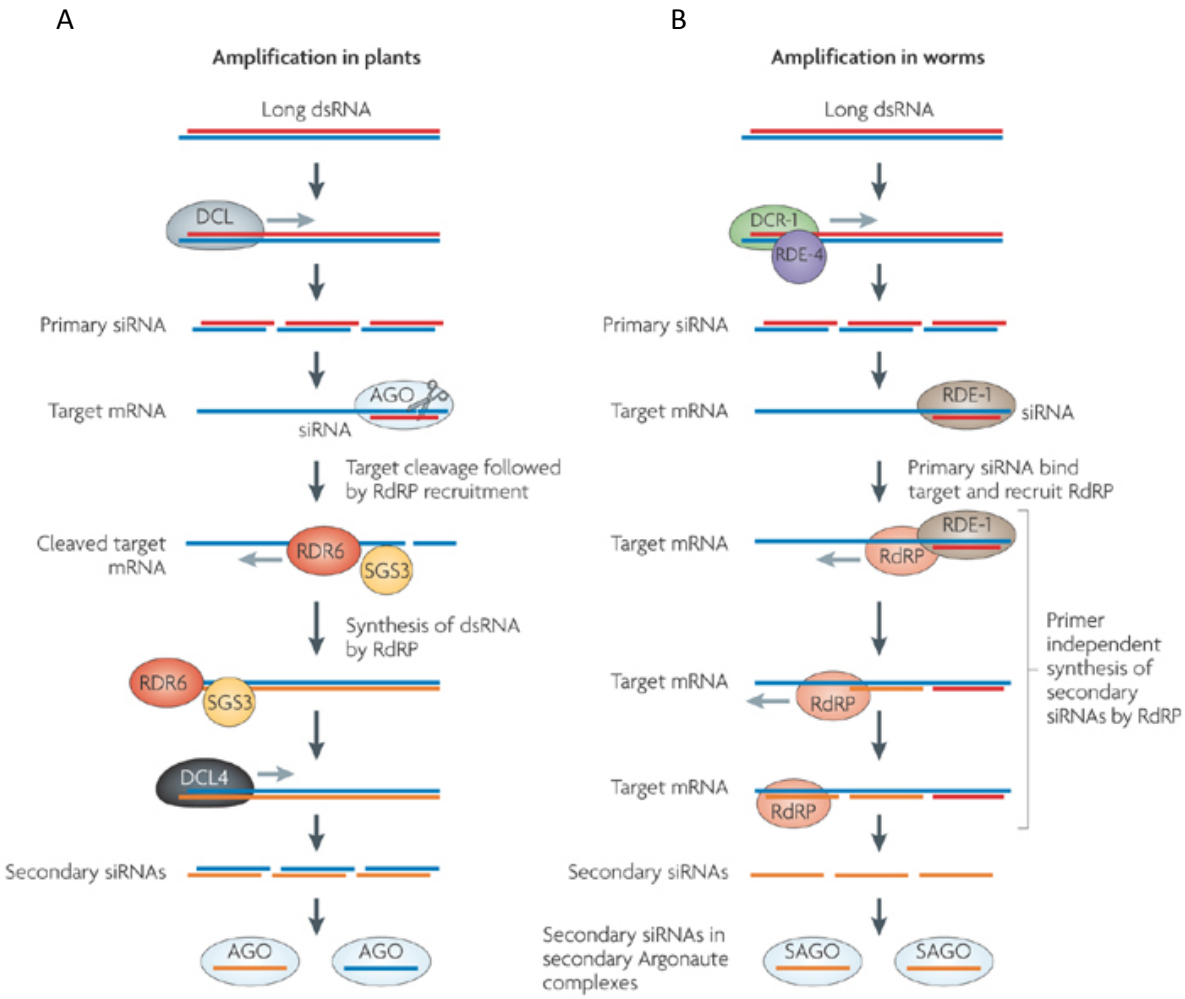


Fig. 6. RNA-dependent RNA-Polymerases (RdRPs) can amplify the silencing response. Primary siRNAs, produced by Dicer like 1 (DCL) in plants and Dicer-1 (DCR-1) in *C. elegans*, bind their mRNA targets and direct cleavage by Argonaute (AGO). (A) In plants, RdRPs (including RDR6) will recognize the cleaved templates and synthesize a complementary antisense strand. The resulting long dsRNA-fragments are diced into secondary siRNAs by Dicer like 4 (DCL4), which in turn will direct cleavage of the target transcripts by AGO. (B) In *C. elegans*, primary siRNAs are bound by RDE-1 (a primary argonaute). After binding to the target mRNA, RDE-1 recruits RdRPs that will produce secondary siRNAs. Secondary siRNA production does not require Dicer, but directs secondary argonaute (SAGO) complexes to new target mRNAs. Image credit: Ghildiyal and Zamore (2009).

1.6. RNA interference in insects

Insects are the most diverse group of animals on the planet, with over 1 million species described. Since they are one of the major contributors to biodiversity in the majority of habitats, with the exception of the sea, which is dominated by another arthropod group, the crustaceans, human welfare is influenced in many ways: (1) domestic insects have an huge economic value by production of popular products (such as silk, honey, wax, etc.) and pollination of plants (Losey and Vaughan, 2006). (2) Insects are also the largest group of food competitors for mankind, causing an enormous reduction in global crop production. Losey and Vaughan (2006) calculated that this would correspond to a loss of $ 15.76 billion per year for the U.S.. (3) By spreading diseases, such as malaria, sleeping sickness, elephantiasis and river blindness, insects are considered as one of the major vectors of human diseases. As an illustration, according to the World Health Organization between 660 000 and 1.2 million people died from the malaria disease in 2010. (4) Finally, from a scientific molecular point of view, many insects are interesting genetic model organisms (small, short generation time and large offspring) and are by their great diversity ideal for comparative studies of physiology, evolutionary biology, developmental biology and population biology.

Keeping in mind the fact that RNAi can be used for functional biological research, for the development of insect-specific insecticides and to inhibit viral replication in (vector and beneficial) insects, RNAi has great potential to positively influence the interaction between man and insects.

1.6.1. Biological functions of small RNAs in insects

Insect smRNAs have been found to participate in multiple biological processes, including development, metabolism, circadian rhythm, insect defence and transposon activity suppression.

1.6.1.1. The antiviral role of siRNAs

Studies have shown that siRNA-directed RNAi plays a clear role in anti-viral immunity in invertebrates and plants. In contrast, mammals have maintained the RNAi-pathway to control endogenous gene expression (via miRNAs), but it is thought that they have

lost the antiviral silencing activity and have instead developed the antiviral interferon response pathway, which is interestingly also triggered by dsRNA. Flies mutant in *dcr2*, *r2d2* or *ago2* have increased sensitivity towards several (ds)RNA viruses, including the Flock House virus (FHV), *Drosophila* C virus, Cricket Paralysis virus, Sindbis virus, Vesicular Stomatitis virus, *Drosophila* X virus, West Nile virus, and Rift Valley Fever virus (Li *et al.*, 2002; Galiana-Arnoux *et al.*, 2006; van Rij *et al.*, 2006; Wang *et al.*, 2006; Zambon *et al.*, 2006b; Chotkowski *et al.*, 2008; Sabin *et al.*, 2010). In addition, it has been demonstrated that the RNAi-pathway can also repress viral replication in mosquitos, including replication of the O'nyong-nyong virus, Sindbis virus, and Dengue virus (Keene *et al.*, 2004; Campbell *et al.*, 2008; Myles *et al.*, 2008; Sanchez-Vargas *et al.*, 2009). Furthermore, RNAi can also provide antiviral defence against DNA viruses, presumably due to the production of dsRNA-fragments by base pairing of convergent overlapping transcripts from both strands of the DNA genome. For instance, this has been demonstrated for the invertebrate iridescent virus 6 (Bronkhorst *et al.*, 2012). Thus, RNAi seems to be the predominant antiviral defence mechanism in insects that provides protection against all major classes of viruses. Furthermore, blocking the endocytotic dsRNA-entry route in *Drosophila* rendered these flies reduced immunity against viral infections. Therefore, the authors proposed that the systemic spread of RNAi could transform distant, uninfected tissues to an antiviral state (Saleh *et al.*, 2009).

The insect RNAi-components and viruses appear to be coevolving, since the RNAi-components *dcr2*, *r2d2* and *ago2* are among the fastest evolving genes in the *Drosophila* genomes (Obbard *et al.*, 2006). Moreover, several insect viruses encode suppressors for the RNAi-response, suggesting that this pathway exerts significant selective pressure on the viruses (Berry *et al.*, 2009).

1.6.1.2. The miRNA-pathway regulates different physiological processes

A genome-wide prediction of miRNA genes by computational methods showed that, in *D. melanogaster*, the ratio of miRNA genes to protein encoding genes is approximately 1:100 (Lai *et al.*, 2003). Evidence from microarray studies suggests that a single miRNA can target hundreds of different mRNAs, indicating that miRNAs regulate a large fraction of protein-encoding genes (Brennecke *et al.*, 2005). This is possible because most miRNAs have limited base pairing with their target, containing

short complementary sequence stretches interrupted by gaps and mismatches (He and Hannon, 2004).

These miRNAs have been demonstrated to regulate important biological processes, including aging, apoptosis, development, cell division, metabolism, neurodegeneration and Wnt/wingless signalling in *Drosophila* (reviewed in (Lucas and Raikhel, 2013)). Although most of the functions and targets of miRNAs have been deciphered in *Drosophila*, recent functional analyses of miRNAs in non-drosophilid insects has begun. Several studies have shown stage-specific or tissue-specific expression of miRNAs during insect development in *Aedes aegypti* (Diptera) (Behura and Whitfield, 2010), *Culex quinquefasciatus* (Diptera) (Skalsky *et al.*, 2010), *B. mori*, *Spodoptera litura* (Lepidoptera) (Yu *et al.*, 2008; Rao *et al.*, 2012), *Blatella germanica* (Blattodea) (Cristino *et al.*, 2011), and the brown planthopper *Nilaparvata lugens* (Hemiptera) (Chen *et al.*, 2012). In addition, a role in phenotypic plasticity has been suggested, with differential expression levels for several miRNAs in forager and nurse heads of the honeybee, *A. mellifera* (Greenberg *et al.*, 2012; Liu *et al.*, 2012a), in different morphs of the pea aphid, *Acyrtosiphon pisum* (Hemiptera), (Legeai *et al.*, 2010) and in the solitary and gregarious phases of the migratory locust, *L. migratoria* (Wei *et al.*, 2009). Moreover, in the cockroach *B. germanica*, silencing *dcr-1* exhibited deep alterations in oocyte development, thus resulting in sterile females (Tanaka and Piulachs, 2012) and generated severe problems during metamorphosis (Gomez-Orte and Belles, 2009). Finally, miRNAs may also contribute to antiviral immunity, since multiple miRNAs are either up- or downregulated upon viral infection in mosquitos (Skalsky *et al.*, 2010).

1.6.1.3. piRNAs in the control of mobile genetic elements

Studies with *Drosophila* Piwi mutants have pointed to a conserved function of piRNAs in the control of mobile genetic elements. Mutations in the *flamenco* locus, which is a piRNA cluster, resulted in defects in germ and follicle cell development, and complete sterility (Brennecke *et al.*, 2007). Furthermore, a direct role for *Drosophila* PIWI and AUB proteins in germ cell specification during early embryogenesis, germ cell formation, stem cell maintenance and regulation of the oogenesis has also been proposed (Thomson and Lin, 2009).

1.6.2. Species- and tissue-dependency of RNAi

To date for more than 30 insect species, belonging to a variety of different orders, such as Diptera, Hymenoptera, Hemiptera, Lepidoptera, Coleoptera, Isoptera, Neuroptera and Orthoptera, positive sysRNAi-results have been obtained (Belles, 2010). Yet, different insect taxa show diverse degrees of sensitivity to RNAi. Low sensitivity to injected dsRNA is widely reported in dipterans and lepidopterans. For example, in larvae of *D. melanogaster*, all cell types, with the exception of the hemocytes, are refractory towards sysRNAi (Miller *et al.,* 2008). In the lepidopterans *Bicyclus anynana*, *Chrysodeixis includes* and *Spodoptera littoralis*, injection of large quantities of dsRNA was ineffective to induce RNAi (Marcus, 2005; Miller *et al.*, 2008; Iga and Smagghe, 2010; Terenius *et al.*, 2011) and although gene silencing has been reported in *B. mori* and *Manduca sexta*, injection of high quantities of dsRNA (>1 µg/mg tissue) were needed (Terenius *et al.*, 2011). In contrast, many members of the Isoptera, Orthoptera and Coleoptera seem to be highly responsive towards injection of dsRNA, with effective dosages as low as 5 pg/larva reported for *T. castaneum* (Bucher *et al.* 2002).

Besides species-dependency of RNAi, tissue-dependency has also been reported. For instance, it was shown that silencing the lipophorin receptor in *B. germanica* occurred faster in the fat body than in the ovary (Ciudad *et al.*, 2007), in *Anopheles gambiae* (Diptera), gene silencing was less effective in the salivary glands than in the midgut and ovarian tissues (Boisson *et al.*, 2006), and also in the nematode, *C. elegans*, tissue-dependency was reported, with the central nervous system (CNS) being less responsive (Timmons *et al.*, 2001; Kamath *et al.*, 2003). In addition, induction of RNAi in *C. elegans* germ cells required additional transport systems (rsd-2, -3 and -6) that were not required for RNAi in somatic cells (Tijsterman *et al.*, 2004).

In *D. melanogaster*, injection of dsRNA is ineffective to induce RNAi, with the exception of the sysRNAi-sensitive hemocytes (Miller *et al.,* 2008). On the other hand, intracellular expression of dsRNA-molecules can successfully induce gene silencing in most *Drosophila* tissues, suggesting that the limiting step is the delivery of intact dsRNA to the cells. Yet, differential RNAi-sensitivity may in some species also be a result of differences in the expression levels of core RNAi-components. This was illustrated for *A. gambiae*, where the less responsive salivary glands display reduced expression levels of *dcr* and *ago* transcripts (Boisson *et al.*, 2006). Interestingly, low

transcript levels of the core RNAi-components, *Bm-R2D2*, *Ms-dcr2* and *Ms-ago2*, have previously been considered as an explanation for the apparently inefficient RNAi-responses in *B. mori* and *M. sexta*, respectively (Swevers *et al.*, 2011; Garbutt and Reynolds, 2012).

An alkaline nuclease that can digest dsRNA has been isolated from the digestive juice of silkmoth larvae. In fact, it can also degrade DNA, although to a lower degree. The nuclease is characterized by the presence of a single DNA/RNA non-specific nuclease (NN)-domain that is preceded by a spacer region and a signal peptide (Arimatsu *et al.*, 2007). In addition, dsRNase-activity has recently been demonstrated in the gut juice of the migratory locust, *L. migratory* (Luo *et al.*, 2013) and in the saliva of the plant bug, *Lygus lineolaris* (Hemiptera) (Allen and Walker, 2012). It was suggested that this might account for the failure of gene silencing upon feeding dsRNA. Furthermore, the success of RNAi might also be influenced by extracellular dsRNA-degrading enzymes in the hemocoel of insects and has previously been proposed as an explanation for the resistance of *M. sexta* towards injected dsRNA (Garbutt *et al.*, 2013). On the other hand, the reduced RNAi-sensitivity of the *C. elegans* CNS is though to be due to lower expression levels of the SID1 dsRNA-transporter and by higher expression levels of the dsRNase ERI1 that degrades intracellular dsRNA (Kennedy *et al.*, 2004; Calixto *et al.*, 2010).

Taken together, the differences in susceptibility for RNAi in insects may be due to differences in the dsRNA-stability in the body cavity (or the gut lumen), penetrance of the cells and tissues, sensitivity of the core RNAi-machinery and regulation of the intracellular dsRNA-levels via dsRNA-degradation.

1.6.3. Lipophorins

Lipoproteins can mediate the transport of lipophilic compounds in a hydrophilic environment. Whereas mammals rely on a battery of lipoproteins (high density lipoproteins (mHDL), low density lipoproteins (mLDL), very low density lipoproteins (mVLDL) and chylomicrons) to effect lipid transport, insects use a single type of lipoprotein, termed lipophorin, for the transport of lipids. The insect lipophorins can be further subdivided into high density lipophorins (HDL) and low density lipophorins (LDL) according to their buoyant density range. Two proteins have been observed in all lipophorins, Apolipophorin 1 (Apo1) and Apolipophorin 2 (Apo2),

with a molecular mass of 220 – 250 kDa and 70 – 85 kDa, respectively. In LDL, and in some adult HDL, a third exchangeable Apolipophorin (Apo3) can also be found (reviewed in Rodenburg and Van der Horst, 2005). The function of Apo3 is to transport lipids (especially diacylglycerol (DAG)) from sites of lipid storage in the fat body to sites of utilization of energy, e.g. flight muscles. During periods of high energy demand, triacylglycerols (TAG) will be converted into DAG, which will leave the fat body and associate with the HDL. Therefore, the HDL is converted into a LDL and Apo3 becomes associated to stabilize the LDL structure. At the site of energy demand, e.g. flight muscles, a lipoprotein lipase will hydrolyse the DAG. The LDL is converted into a HDL and cycles back to the fat body, where, once again, it will be converted into the low-density form (Derewenda, 1994; Rodenburg and Van der Horst, 2005).

In line with the concept of selective lipid transfer, lipoproteins are also internalized by means of (clathrin-dependent) receptor-mediated endocytosis. Two types of receptors have been identified in lipophorin transport, the lipophorin receptor (LpR) and SRs. The LpR has a 8-fold higher affinity for DAG-rich lipophorins (LDL) than for DAG-poor lipophorins (HDL) (Derewenda, 1994), while SRs have the highest affinity for modified (acetylated or oxidized) lipoproteins (Krieger and Herz, 1994).

Whereas, mammalian lipoproteins are also typically characterized as a shuttle for dietary energy, more recent studies illustrate that they also play an important role in innate immunity by scavenging for bacterial, fungal and viral pathogen-associated molecular patterns (Han, 2010). Likewise, in insects, Apo3 has been found to be an key player in defence against fungal (Whitten *et al.*, 2004) and bacterial pathogens (Gotz *et al.*, 1997; Dettloff *et al.*, 2001; Cheon *et al.*, 2006; Ma *et al.*, 2006). In the hemolymph of *B. mori*, lipophorins were found to bind to dsRNA. Moreover, several studies have demonstrated that the mammalian (m)LDL possesses DNA- and RNA-binding activity (Guevara *et al.*, 1999). Moreover, it has been shown that mLDL can be used to transport and deliver plasmid DNA to the cell nucleus (Guevara *et al.*, 2010). Although the underlying nucleic acid binding activity is not fully understood, the presence of Arg/Lys-rich clusters in mammalian Apolipoprotein (mApo)B (which is related to the insect Apo1 and Apo2) and mApoE (which is related to the insect Apo3) might well serve as nucleic acid binding site (Guevara *et al.*, 2010).

1.6.4. RNAi in insect pest management

Insect pests cause severe economic losses in crop production. In the past 60 years, increasing crop yield has relied heavily on pest control through the use of synthetic insecticides. Yet, a lot of problems associated with the application of pesticides have also shown up, including environmental pollution, side effects on non-target organisms, revival of the pest populations due to disruption of the natural control, the evolution of resistance of the target pests and increasing cost.

Therefore, much attention has been paid to the development of alternative insect pest management strategies, with reduced side effects. This has led to the use of natural enemies, natural product based pesticides, pest-resistant genetically modified (GM) crops and combinations of these strategies. One of the most successful examples is the use of the Crystal (Cry) proteins produced by *Bacillus thuringiensis* (Bt). These Bt-toxins kill certain insect groups, such as mosquitos, caterpillars and beetles, but are harmless to vertebrates and most other organisms (de Maagd *et al.*, 2001). Yet, insect resistance to Bt-toxins in laboratory and field populations pose a threat to the effectiveness of Bt-toxins (Tabashnik *et al.*, 2008; Bagla, 2010) and the composition of the pest population has been found to shift from the single dominant insect pest species towards a more diverse population of pest invertebrates (that are more resistant towards Bt-toxins), as a consequence of the Bt-toxin exposure (Lu *et al.*, 2010).

The potential of RNAi in the development of alternative insect control methods, was first demonstrated by Baum *et al.* (2007) that conferred corn plants resistance against the western corn rootworm (WCR), *Diabrotica virgifera* (Coleoptera), through the generation of transgenic plants that produce dsRNA targeting the WCR vacuolar ATPase gene. This was followed by an alternative approach by Mao *et al.* (2007) who developed GM-plants that synthesize dsRNA against the defence related P450 gene, resulting in reduced tolerance of the cotton bollworm, *Helicoverpa armigera* (Lepidoptera), against the toxin gossypol produced by cotton plants. Consequently, this generated high mortality rates in the pest insect population. To date, oral delivery of dsRNA has also been proven to be feasible in several other insect species, including in *Bactrocera dorsalis* (Diptera) (Li *et al.*, 2011), *A. mellifera* (Hunter *et al.*, 2010), *Epiphyas postvittana* (Lepidoptera) (Turner *et al.*, 2006), *S. frugiperda* (Griebler *et al.*, 2008), *Plutella xylostella* (Lepidoptera) *(Bautista et al., 2009)*, *M. sexta*

(Kumar *et al.*, 2012), *Rhodnius prolixus* (Hemiptera) (Araujo *et al.*, 2006), *A. pisum* (Pitino *et al.*, 2011; Mao and Zeng, 2012), and *Reticulitermes flavipes* (Isoptera) (Zhou *et al.*, 2008b).

Regarding RNAi-based insect pest control, one of the major concerns is to avoid cross-species off-target effects. Wong and co-workers successfully avoided the cross-species effects when they silenced *γ-tubulin 23C* in four *Drosophila* species (*D. melanogaster*, *D. sechella*, *D. yakuba* and *D. pseudoobscura*) (Whyard *et al.*, 2009). Since *Drosophila* is in general insensitive towards sysRNAi, the dsRNA was introduced into the cells through lipofection. Although the *γTub23C* gene is highly conserved, the untranslated region (UTR) displays lower sequence similarity between the four homologous. Delivery of dsRNA targeting the UTR of *γTub23C* affected only the viability of the conspecific organisms, suggesting that selection of less conserved genes (or fragments) could improve the species-specificity.

Another problem to consider is the fact that RNAi does not kill the insect pest immediately. As a consequence, the possibility that insects currently sensitive to RNAi will evolve resistance cannot be excluded and, regarding the high variance in sensitivity towards sysRNAi, the pest population might shift towards less responsive insects (Tabashnik *et al.*, 2005). On the other hand, keeping in mind the fact that the cell-autonomous RNAi-response plays a crucial role in antiviral immunity and development, the development of delivery agents that can transfer the dsRNA directly into the gut cells holds great promise.

1.6.5. RNAi-based control of viral spread

Many viruses, which fall under the generic name “arboviruses”, are transmitted to vertebrates by infected arthropods (mainly by mosquitos and ticks). Among many more, arboviruses include the dengue virus, Japanese encephalitis virus, West Nile virus, tick-borne encephalitis virus, yellow fever virus, and Rift Valley fever virus. The most prevalent mosquito-borne viral disease is dengue, with an estimate of 100 million infections each year, 250 000-500 000 of which cause severe illness (Gould and Solomon, 2008). In addition, insects (especially Hemiptera) constitute important vectors for plant viruses. These and other plant viruses cause an estimated US$60 billion loss in crop yields worldwide each year (Wei *et al.*, 2010), as well as sever losses for forestry. Furthermore, the livelihood of several beneficial insects, such as

honeybees and bumblebees, is threatened by viral infections, such as by the Israeli Acute Paralysing Virus (IAPV) and the deformed wing virus (Maori *et al.*, 2009).
In this context, delivery of dsRNA molecules against these viruses has potential to reduce viral loads in vector and beneficial insects. For instance, as a proof of concept, it has been shown that expression of an inverted repeat construct targeting the dengue virus-2 RNA genome significantly reduced the transmission of the virus (Franz *et al.*, 2006), and feeding honeybees with dsRNA fragments that target the IAPV resulted in larger colony populations and thus increased honey production (Hunter *et al.*, 2010).

1.7. Aim of this study

Since many economically important insect species display limited sensitivity towards sysRNAi, the RNAi-technology is currently not used to its full potential. At the start of this PhD-project, the mechanisms of RNAi in insects were almost exclusively based on studies in *D. melanogaster*, which is characterized by a low sensitivity towards sysRNAi. On the other hand, our research group demonstrated that the desert locust, *S. gregaria*, responds very well towards injected dsRNA (Badisco *et al.*, 2011b; Marchal *et al.*, 2012; Ott *et al.*, 2012, Van Wielendaele *et al.*, 2012). Therefore, in this PhD-project, we further studied the characteristics and mechanisms of sysRNAi in this RNAi-sensitive insect species, with the aim to identify effector mechanisms that determine the success of sysRNAi in insects. In this respect, (i) dsRNA-degrading and –binding proteins in the hemocoel and gut lumen, (ii) cellular dsRNA-uptake mechanisms and (iii) components involved in the cell autonomous RNAi-machinery were identified. Moreover, our study brings in new fundamental insights in how the potency of the RNAi-response might be regulated and could lead to new strategies to improve gene-silencing techniques in other, less responsive insect species. Furthermore, regarding the economical value of *S. gregaria* as a voracious agricultural pest insect and research model organism, our data concern the potential of RNAi to control locust populations and improve the usage of RNAi in loss-of-function studies in locusts.

Chapter 2

General Materials and Methods

Chapter 2: General Materials and Methods

2.1. Rearing of *S. gregaria*

Gregarious *S. gregaria* were reared under crowded conditions with controlled temperature (32±1°C), light (13h photoperiod) and relative humidity (40-60%). The locusts were kept at high density (> 200 locusts/cage) in special wooden cages and fed daily with fresh cabbage and dried oat flakes. Mature female locusts deposited their eggs in pots with moistened sterile sand mixture (7 parts sand, 3 parts peat and 1 part water). These pots were collected once a week and set apart in empty cages, resulting in pools of 1st instar locusts, which differ maximum 1 week in age. To synchronize the locusts, they were separated directly after their final moult.

2.2. *S. gregaria* Expressed Sequence Tag (EST)-databases

In 2005, our laboratory constructed in collaboration with W.M. Keck Centre for Comparative and Functional Genomics (University of Illinois, Urbana-Champaign, U.S.A.), a normalized cDNA library from dissected Central Nervous Systems (CNS) of solitarious and gregarious, and larval and adult desert locusts, males and females. From in total 34 672 raw EST data with for most sequences a length of 600-900 nucleotides of high quality sequence information (>99% confidence level), 12 709 unique sequences were retrieved. The data have been integrated in a desert locust 'EST information Management and annotation' (ESTIMA) database (http://titan.biotec.uiuc.edu/locust/) (Badisco *et al.*, 2011a).

In *D. melanogaster*, *dicer-2* (*dcr2*) (NM_079054), *argonaute-2* (*ago2)* (NM_140518.3), *clathrin heavy chain* (*clath*) (NM_057694.2) and *vacuolar H-ATPase 16* (*vha16*) (NM_057453.5) were identified to participate in the siRNA-directed gene silencing verified (Kim *et al*., 2006; Saleh *et al.*, 2006). Therefore, these sequences were used as query to retrieve the homologous *S. gregaria* transcripts from the annotated *S. gregaria* EST-database. The *C. elegans exoribonuclease 1* (*eri-1*) (NM_171245.4) , whose function was experimentally validated, was used as query to find the *S. gregaria eri1* transcript sequence.

On the other hand, the identity of the *S. gregaria glyceraldehyde phosphate dehydrogenase* (*gapdh*), *alpha-tubulin 1a* (*tubu*), *ubiquitin conjugating enzyme 10*

(*ubi*), *elongation factor 1a* (*ef1a*), *β-actin*, *CG13220* and *ribosomal protein* (*rp*)*49* was previously verified by Van Hiel *et al.* (2009).

2.3. *S. gregaria* transcriptome database

In 2012, our research group started with the construction of a transcriptome database from entire 5th instar desert locusts and from muscle, salivary gland, gut, reproductive system and CNS tissue of adult *S. gregaria*, both sexes, and both phases. The transcripts were sequenced using the Illumina hiseq 2000 sequencing system and Roche 454. The assembly also included *S. gregaria* transcript sequence data from the Max Planck Institute for Chemical Ecology (ICE, Department of Entomology, Jena), the German Cancer Research Centre (Epigenetics, Heidelberg) and the research group of Functional Genomics and Proteomics (KU Leuven). Although the assembly (using Velvet software) remains largely incomplete, currently, the *S. gregaria* transcriptome database contains 837 297 different contigs with an average of length 1075 bp.

The transcript sequence information of *S. gregaria dsRNase1*, *dsRNase2*, *dsRNase3*, *dsRNase4* was retrieved from the *S. gregaria* transcriptome database by using the deduced amino acid sequence of the *B. mori* alkaline nuclease (NM_001098274.1), while the *D. melanogaster endonuclease G* (*endoG*) (NM_140819.2) was used to find the *Sg*-endoG transcript sequence. In addition, the *Sg-apolipophorin 1/2 precursor* (*apo1/2*), *Sg-apolipophorin 3* (*apo3*) and *Sg-lipophorin receptor* (*lpr*) were retrieved from the *S. gregaria* transcriptome database by using the homologous sequences of *L. migratory* (AJ130944; J03888.1 and AJ000010.1, respectively). Finally, the *sid-1 like* sequence of the desert locust was retrieved from the transcriptome database by using the *sid1 related C* (*sirC*) sequence of *T. castaneum* (NM_001105658.1).

2.4. Synthesis of dsRNA

Double stranded RNAs for *tubu* (545 bp), *gapdh* (447 bp), *gfp* (589 bp), *dcr2* (330 bp), *ago2* (461 bp), *dsRNase1* (576 bp), *dsRNase2* (346 bp), *dsRNase3* (496 bp), *dsRNase4* (646 bp), *apo1/2* (631 bp), *apo3* (277 bp), *clath* (561 bp), *vha16* (453 bp), *lpr* (520 bp), *sid1 like* (250 bp) were synthesized using the MEGAscript RNAi kit (Ambion). With the exception of *gfp*, a DNA template flanked by two T7 promoter sequences was generated. A PCR reaction was performed using adult desert locust cDNA and gene-

specific primers containing a T7 promoter sequence at the 5' end. In table 1, the different PCR primers are displayed. REDTaq mix (Sigma-Aldrich co.) was used as a source of DNA Taq polymerase, dNTPs and PCR buffer. Next, the amplification products were analysed by 1% agarose gel electrophoresis and then visualized with UV-light (Proxima). Finally, bands of the desired size were excised and purified using GenElute™ Gel extraction Kit (Sigma–Aldrich Co.). The amplified DNA fragments were cloned into the pCR®4-TOPO® vector by means of the TOPO TA Cloning® Kit for Sequencing (Invitrogen). The sequences of the inserted DNA fragments were determined using the ABI PRISM BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems).

A TOPO 4.1 sequencing vector (Life technologies Co.) containing a *gfp* transcript sequence was used as template for *gfp* dsRNA production. Since only one T7 promoter site is present in this vector, the fragment was cloned both in the sense and antisense direction. RNA was then synthesized by the T7 Enzyme Mix of the MEGAscript RNAi kit (Ambion). Both *gfp* RNA strands were first synthesized independently before being mixed to anneal, while transcripts made from a single template with opposing T7 promoters were present in the same mixture. After the production of dsRNA, the remaining DNA and ssRNA was removed by nuclease treatment, and proteins and mono/oligonucleotides were removed by solid phase adsorption purification, according to the manufacturers' specifications (Ambion). The dsRNA-concentration was determined with a Nanodrop spectrophotometer (Thermo Fisher Scientific), and the integrity of the dsRNA was assessed with gel electrophoresis using a 1% agarose gel. The dsRNA was stored at -20°C until further usage.

	Fw-primer	Rv-primer
tubu	**TAATACGACTCACTATAGGG** ATTTTTTAGCGAAACTGGTGCTGGG	**TAATACGACTCACTATAGGG** TGGTGTAAGTCGGGCGTTCAATGT
gapdh	**TAATACGACTCACTATAGGG** CCGTTGCTGTCGGTTCGTAGGAA	**TAATACGACTCACTATAGGG** TTGGGGCATCTGCACTTGGA
dcr2	**TAATACGACTCACTATAGGG** GCTGATGCTCTTGAGGCTCT	**TAATACGACTCACTATAGGG** TGCATCACCAAGAAATTCCA
ago2	**TAATACGACTCACTATAGGG** TTCCGCTAGGAAATGGAATG	**TAATACGACTCACTATAGGG** GCACAGCTCCACTGGTAAG
dsRNase1	**TAATACGACTCACTATAGGG** CCTTTCCAAAGGACACCTCA	**TAATACGACTCACTATAGGG** CCTGCGGGTTTTTGAAGATA
dsRNase2	**TAATACGACTCACTATAGGG** GGACACCTCGCTGCTAAGTC	**TAATACGACTCACTATAGGG** CGATGTACGGATTGTTGACG
dsRNase3	**TAATACGACTCACTATAGGG** GCTAGCGGTAGCAGGAACAC	**TAATACGACTCACTATAGGG** ACTGCCCACCACAGTACCTC
dsRNase4	**TAATACGACTCACTATAGGG** TCGTCTACGAGCCGAAATCT	**TAATACGACTCACTATAGGG** TCGTGGATCACTTGCTAACG
apo1/2	**TAATACGACTCACTATAGGG** TTTGAGATTGCTGCACGAAC	**TAATACGACTCACTATAGGG** CATCAACAAGCAGCTTTCCA
apo3	**TAATACGACTCACTATAGGG** CAGGTGAACATCGCAGAGAC	**TAATACGACTCACTATAGGG** GGTGCCCTGGTGCTTCTC
clath	**TAATACGACTCACTATAGGG** AAATCTTGATCGGGCATACG	**TAATACGACTCACTATAGGG** CAATGTGAAGGCCACA
vha16	**TAATACGACTCACTATAGGG** TGCGACCAGAGCTGATTATG	**TAATACGACTCACTATAGGG** GAGGTGGCTGCTGTAGGAAG
lpr	**TAATACGACTCACTATAGGG** CAGTCTTGGGTGTGTGATGG	**TAATACGACTCACTATAGGG** CCTCAACGCATTGACATTTG
sid1 like	**TAATACGACTCACTATAGGG** TGTCATCTTGATTGGCATGG	**TAATACGACTCACTATAGGG** ACAAGTTACCCAGCGTGAGC

Table 1. Primer sequences to make a DNA template flanked by two T7 promoter sites for production of dsRNA targetting *S. gregaria alpha-tubulin 1a* (*tubu*), *glyceraldehyde phosphate dehydrogenase* (*gapdh*), *dicer-2* (*dcr2*), *argonaute-2* (*ago2), dsRNase1, dsRNAse2, dsRNase3, dsRNAse4, apolipophorin 1/2 precursor* (*apo1/2*), *apolipophorin 3* (*apo3*), *clathrin heavy chain* (*clath*), *vacuolar H-ATPase 16* (*vha16*), *lipophorin receptor* (*lpr*), *systemic RNA interference 1 like* (*sid1 like*).

2.5. Injection and feeding of dsRNA

Locusts were intra-abdominally injected, orally injected or fed with dsRNA-containing medium. *S. gregaria* (*Sg*-)Ringer solution (1 L: 8.766 g NaCl; 0.188 g $CaCl_2$; 0.746 g KCl; 0.407 g $MgCl_2$; 0.336 g $NaHCO_3$; 30.807 g sucrose; 1.892 g trehalose; pH 7.2) was used to dilute the dsRNA to the desired concentration.

(i) Fifth larval stage locusts were each intra-abdominally injected with a volume of 6 µl of dsRNA-solution and adult locusts with a volume of 10 µl. (ii) Adult desert locusts were also orally injected with 5 µl dsRNA-solution using a pipet. Therefore, the locusts were first starved for one day and after the oral dsRNA-injection they were put directly on a cabbage leaf. (iii) In addition, an artificial medium (2.4% wesson salt, 0.5% linoleic acid, 0.6% cholesterol, 0.3% ascorbic acid, 0.2% Vanderzant vitamin cocktail (Sigma-Aldrich co.), 54% cellulose, 14% sucrose, 14% dextrin and 14% protein (3:1:1 casein, peptone and albumin)) was used to feed dsRNA to the locusts. To solidify the medium, agarose was added to generate a 0.5 % agarose gel. The mixture was heated in a microwave and, after cooling the samples to approximately 40°C, the dsRNA was added to the mixture. The locusts were starved for one day and subsequently separated to individual cages and a small (cubes with sides measuring approximately 0.5 cm) quantity of the artificial diet, with dsRNA added, was daily delivered to the locusts (for four times). Locusts that did not eat the entire diet were discarded from the experiment.

2.6. Tissue collection

The locust tissues of interest were micro-dissected in *Sg*-Ringer solution under a binocular microscope. Tissues that were dissected for the purpose of RNA-extraction with the Lipid tissue extraction kit (Qiagen; § 2.7) were collected in "MagNA Lyser Green Beads" containing tubes (Roche). Smaller tissues that were further processed by means of the RNAqueous®-micro kit (Ambion; § 2.7) were stored in RNase en RNA free tubes. Next, the tubes were immediately transferred to liquid nitrogen to prevent RNA degradation. All samples were stored at −80 °C until further processing.

2.7. RNA extraction and cDNA synthesis

The Lipid tissue extraction kit (Qiagen) was utilized to extract RNA from larger tissues, such as gut, gonadal, muscle and fat body tissue and all pooled samples, while the RNAqueous®-micro kit (Ambion) was employed to extract RNA from the relatively smaller brain and malpighian tubule samples. This was followed by an additional DNase treatment to remove genomic DNA contamination. Quality and concentration of the extracted RNA were assessed using a Nanodrop spectrophotometer (Thermo scientific, inc.). Next, equal quantities of RNA were used as template to produce cDNA. For the cDNA synthesis in Chapter 3 and 4, we used the Superscript III reverse transcriptase kit (Invitrogen), random hexamer primers (Invitrogen) and dNTPs (Roche diagnostics GmbH) as described in the manufacturer's protocol. Finally, the cDNA-solution was 10-times diluted with MilliQ water. A calibrator cDNA sample was also made based on a pooled tissue sample containing all investigated tissues. On the other hand, the PrimeScript™ First strand cDNA Synthesis Kit (TaKaRa) could also produce cDNA of good quality, yet for a better prize. Therefore, in Chapter 5 and 6 we used the PrimeScript™ First strand cDNA Synthesis Kit (TaKaRa) for the production of cDNA. The procedure was followed according to the manufacturer's specifications and the cDNA-samples were 15-times diluted with MilliQ water. Calibrator samples containing pooled tissues of all investigated samples were also synthesized with this kit. The resulting cDNA-samples were analysed immediately or stored at -20°C until further usage.

2.8. (Optimalization of) quantitative real time-PCR

Primer express software (Applied Biosystems) was used to design quantitative real time (q)PCR primers. The corresponding primer sequences are displayed in Table 2. The primers were validated with a standard curve based on a serial dilution of cDNA to determine the primer annealing efficiency and a dissociation protocol was performed to detect the presence of primer dimers and production of a single PCR product. For all transcripts, only a single melting peak was found. In addition, the qPCR products were run on a 1% agarose gel containing GelRedTM (Biotium) that resulted in a single band. Each reaction was performed in duplicate and contained 10 µl SYBR green solution (Invitrogen), 0.75 µl of 10 µM forward primer (Sigma aldrich),

0.75 µl of 10 µM reverse primer (Sigma Aldrich), 3.5 µl milliQ water and 5 µl cDNA. Seven household genes were chosen, based on the study of Van Hiel and co-workers (Van Hiel *et al.*, 2009), namely *tubu*, *gapdh, ubi*, and *ef1a*, *β-actin*, *CG13220* and *rp49*. The geNorm program (Pattyn *et al.*, 2003) was used to determine the most stable reference genes between the different tissues and time points investigated, namely between fat body, muscle, brain, midgut, malpighian tubules, male and female reproductive systems, for 4 and 10 days old adult desert locusts. The four genes that were most stably expressed were, respectively, *ubi*, *ef1a, gapdh* and *tubu*. In order to correct for sample-to-sample variations, the relative expression levels were normalized against the two most stably expressed reference genes, namely *ubi* and *ef1a*. The data were further normalized against a calibrator cDNA sample to account for variations in the PCR-efficiency in different PCR runs. In every experiment, no-template controls were included to check for possible contaminations and, for each type of tissue, no-RT controls were validated. The PCR reaction was performed and analysed in a 96 well plate and by the StepOne System (ABI Prism, Applied Biosystems). Since the efficiency of the different primers was the same, the relative transcript quantity was calculated according to the delta-delta Ct method.

	Fw-primer	Rv-primer
ubi	GACTTTGAGGTGTGGCGTAG	GGATCACAAACACAGAACGA
ef1a	GATGCTCCAGGCCACAGAGA	TGCACAGTCGGCCTGTGAT
β-actin	AATTACCATTGGTAACGAGCGATT	TGCTTCCATACCCAGGAATGA
CG13220	TGTTCAGTTTTGGCTCTGTTCTGA	ACTGTTCTCCGGCAGAATGC
rp49	CGCTACAAGAAGCTTAAGAGGTCAT	CCTACGGCGCACTCTGTTG
tubu	TGACAATGAGGCCATCTATG	CGCAAAGATGCTGTGATTGA
gapdh	GTCTGATGACAACAGTGCAT	GTCCATCACGCCAC ACT TC
dcr2	GATTCATAGGTTCGTACCAGATGAAA	GGTTGCGGAACGGTGAAAC
ago2	TCGCCAGACCAGCGAAATAT	CTGGAAGGCATCTGGATCGT
eri1	AACAAGCAGACACTAATACAGAAAATGAG	CAGGCGCTTTTTTAGGACATCT
dsRNase1	GCTCATCGACGCCTGCTT	CGGCGACCATCGTGAAGT
dsRNase2	CCGAGACCAACGACTACTACTTCCT	CGCGCCGAGCATGAAATCAGAC
dsRNase3	CGCCCCAGCGACAGACT	CGAGTTGACGCCGAAGAAG
dsRNase4	CCACAGTGGTACGCCTTCAA	AAGTCGCGCACGTCCAA
endoG	TGAACAGGGTTGCACGTCAA	CGATCAAGGCACTCAAATTCAC
apo1/2	CAGTGCAAAGGCAGCATTGA	CAGTGCCGCTTGTCGCTAAT
apo3	GCGCGCCCAGATGCT	TTGACGATCGTGTGGTTCAGTT
clath	AAGGACGCAATGGAGTATGCA	TTTCCAGAAACCACGCAAGTAA
vha16	CTGCATACGGAACTGCAAGT	CATAATCAGCTCTGGTCGATT
lpr	TGAACAGGGTTGCACGTCAA	CGATCAAGGCACTCAAATTCAC
sid1 like	CTTACTGCCCGAAGAGATCACA	CATAGTGTTGAGGGATGCCATAATA

Table 2. The primer sequences for *ubiquitin conjugating enzyme 10* (*ubi*), *elongation factor 1a* (*ef1a*), *β-actin*, *CG13220* and *ribosomal protein* (*rp*)*49, alpha-tubulin 1a* (*tubu*), *glyceraldehyde phosphate dehydrogenase* (*gapdh*), *dicer-2* (*dcr2*), *argonaute-2* (*ago2), exoribonuclease 1 (eri1), dsRNase1, dsRNAse2, dsRNase3, dsRNAse4, endonuclease G (endoG)*, *apolipophorin 1/2 precursor* (*apo1/2*), *apolipophorin 3* (*apo3*), *clathrin heavy chain* (*clath*), *vacuolar H-ATPase 16* (*vha16*), *lipophorin receptor* (*lpr*), *systemic RNA interference 1 like* (*sid1 like*) that were used for qPCR with *S. gregaria* cDNA.

Chapter 3

Parameters influencing the RNAi-efficiency in the desert locust*, Schistocerca gregaria*

This chapter is partially based on a paper published in Insect Biochemistry and Molecular Biology (Wynant *et al.*, 2012).

Chapter 3: Parameters influencing the RNAi-efficiency in the desert locust, *Schistocerca gregaria*

3.1. Introduction

Several studies have shown that double stranded (ds)RNA can initiate the activation of an innate immune response, resulting in the sequence-specific inhibition of cellular gene expression at the post-transcriptional level (Fire *et al.*, 1998). This process, better known as RNA interference (RNAi), has become a widely used reverse genetic tool to knock down and analyse the function of genes (Belles, 2010). Besides functioning as a research tool, RNAi may also contribute to novel strategies for selectively controlling agricultural pests, including a number of insect species (Baum *et al.*, 2007; Mao *et al.*, 2007; Whyard *et al.*, 2009). In contrast to conventional insecticides, RNAi is a nucleic acid sequence dependent process. Consequently, RNAi is a highly species-specific technology, the use of which should result in fewer non-target effects and reduced harm to agricultural ecosystems. However, in order to induce an RNAi-response, dsRNA must enter the cell. Fortunately, some organisms are able to import extracellular dsRNA. In addition, in several plants and nematodes, secondary dsRNA molecules are produced by RNA-dependent RNA-polymerases (RdRPs), whereby even with the presence of very low dsRNA quantities, a robust and persistent RNAi-response will be achieved (Schiebel *et al.*, 1998; Sijen *et al.*, 2001). However, until now no canonical RdRP orthologous sequences have been found in the genome of insects (Belles, 2010).

In many insect species, representing the orders of Lepidoptera, Coleoptera, Diptera, Hemiptera, Hymenoptera, Dictyoptera, Isoptera and Orthoptera, systemic (sys)RNAi-responses have been reported (Belles, 2010). SysRNAi describes the phenomenon in which RNAi is established in tissues distant from the site of dsRNA-administration. In particular, sysRNAi is characterized by the fact that intra-abdominal injection or oral delivery of dsRNA results in gene silencing effects throughout the body. However, the degree of sensitivity toward RNAi varies strongly among insect species (Miller *et al.*, 2008; Belles, 2010; Terenius *et al.*, 2011). The best-known example of a species with a reduced sensitivity towards sysRNAi is the fruit fly *D. melanogaster* (Miller *et al.*, 2008). In its larval stage, only the hemocytes seem to be capable of responding in a

systemic manner. Furthermore, in many Lepidoptera, such as *B. mori*, *M. sexta* and *S. littoralis*, most tissues seem to be rather refractory to a systemic response (Terenius *et al.*, 2011). On the other hand, recent reports suggest that some Orthoptera, Dictyoptera and Coleoptera respond very well to systemically applied dsRNA (Dong and Friedrich, 2005; Miller *et al.*, 2008; Belles, 2010; Badisco *et al.*, 2011b). Most positive RNAi-results have been obtained by injection of the dsRNA. Yet, feeding of dsRNA has also been proven to be feasible in several insect species, including insects that belong to the order of Lepidoptera (Turner *et al.*, 2006; Griebler *et al.*, 2008) Diptera (Li *et al.*, 2011), Hemiptera (Araujo *et al.*, 2006; Pitino *et al.*, 2011; Mao and Zeng, 2012), Hymenoptera (Hunter *et al.*, 2010) and Isoptera (Zhou *et al.*, 2008b).

Here, we report on the first detailed description of the sysRNAi-response in a member of the Orthoptera, namely *Schistocera gregaria*, a voracious pest insect and an important research organism. Although previous studies have proven the presence of a robust sysRNAi-response in *S. gregaria* (Badisco *et al.*, 2011b; Marchal *et al.*, 2012; Van Wielendaele *et al.*, 2012), its sensitivity, persistence, tissue dependency and the feasibility towards orally delivered dsRNA has not been investigated in detail yet and are assessed in this Chapter.

3.2. Materials and Methods

3.2.1. Sequence information

The *alpha-tubulin 1a* (*tubu*) and *glyceraldehyde aldehyde phosphate dehydrogenase* (*gapdh*) transcript sequences were found in the available *S. gregaria* EST-database and, prior to the onset of these experiments, our research group validated these transcript sequences (Van Hiel *et al.*, 2009).

3.2.2. Synthesis of dsRNA

Production of dsRNA for *gapdh* (447 bp), *tubu* (545 bp) and *green fluorescent protein* (*gfp*, 589 bp) were synthesized by using the MEGAscript RNAi kit (Ambion) as described in § 2.4. The primer sequences are also displayed in § 2.4.

3.2.3. Injection or feeding of dsRNA

Four-day old adult locusts were intra-abdominally or orally injected with dsRNA that was diluted to the desired concentration with *Sg*-Ringer solution (1 L: 8.766 g NaCl; 0.188 g $CaCl_2$; 0.746 g KCl; 0.407 g $MgCl_2$; 0.336 g $NaHCO_3$; 30.807 g sucrose; 1.892 g trehalose; pH 7.2), in accordance to the procedure described in § 2.5. An artificial diet containing dsRNA was also used to fed the locusts as described in § 2.5.

3.2.4. RNA-extraction and cDNA-synthesis

The Lipid tissue extraction kit (Qiagen) was utilized to extract RNA from larger tissues, such as gut, gonadal, muscle and fat tissue, while the RNAqueous ®-micro kit (Ambion) was employed to extract RNA from the relatively smaller brain and malpighian tubule samples. This was performed in accordance to the methods described in § 2.7. The RNA-samples were stored at -80°C. Next, equal quantities of RNA were used as template to produce cDNA. The cDNA synthesis was performed using the Superscript III reverse transcriptase kit (Invitrogen), random hexamer primers (Invitrogen) and dNTPs (Roche diagnostics GmbH) as described in § 2.7.

3.2.5. Quantitative real time-PCR

The knockdown was assessed by means of qPCR in accordance to the described procedures and by using the primer sequences described in § 2.8.

3.2.6. Statistical analysis

All data were analysed by non-parametric statistics in GraphPad prism 5 (GraphPad). In addition, the change of transcript knockdown efficiency over time (section 3.2) was assessed by linear regression analysis.

3.3. Results

3.3.1. Tissue-dependence of the systemic RNAi-response

Five µg of *sg-gapdh* dsRNA was injected into the haemocoel of four-day old adult locusts. Three days later, the tissue-dependent knockdown was determined by comparing the relative *gapdh* transcript level between the test and control group, *i.e.*

locusts injected with *gfp* dsRNA. The data (Fig. 1) indicate that a very robust knockdown was achieved for most tissues investigated, with the exception of the male and female reproductive systems. While a significant knockdown was still observed in the reproductive system, it was tempered in comparison to the other tissues investigated.

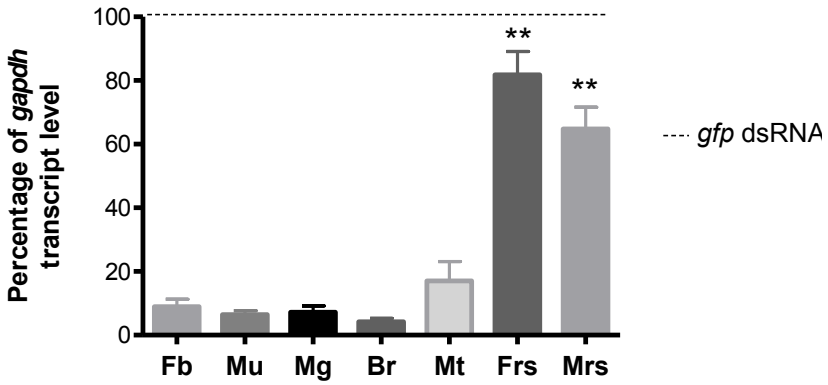


Fig. 1. Tissue-dependence of sysRNAi, three days after injection of 5 µg of dsRNA. Seven different tissues were examined: Fb (fatbody), Mu (muscles), Mg (midgut), Br (brain), Mt (malpighian tubules), Frs (female reproductive system) and Mrs (male reproductive system). Significantly higher transcript levels were observed in the Mrs and Frs. The knockdown is represented as the mean percentage of the relative *gapdh* transcript levels in the test group in comparison to the mean relative transcript levels in the control groups that were injected with *gfp* dsRNA (mean ± SEM, n = 5-6, **: $p < 0.01$).

3.3.2. dsRNA-dose and time dependence of the RNAi-response

The dsRNA-dose dependence was studied by intra-abdominal injection using various quantities of *gapdh* dsRNA. After three days, the knockdown for two distinct tissues was examined: the female reproductive system, which is known to be more resistant towards RNAi, and the brain, which is one of the more responsive tissues (§ 3.3.1). The data demonstrate that the RNAi-response was extremely sensitive (Fig. 2A). Injection of only 1 ng of *gapdh* dsRNA was sufficient to induce a significant knockdown. Moreover, the *maximum concentration dependent silencing* was observed upon administration of as little as 30 ng dsRNA, a quantity that corresponds to an average of 15 pg per mg tissue. Remarkably, despite the reduced knockdown robustness in the female reproductive system, both tissues displayed a similar

dsRNA-dose-response relationship. To ascertain that these observations were not specific for *gapdh* gene silencing, a similar experiment was conducted using *tubu* dsRNA. Silencing of *tubu* resulted in a similar dose-dependent response (Fig. 2B), suggesting that the reported sensitivity is not specific to the *gapdh* gene or dsRNA sequence.

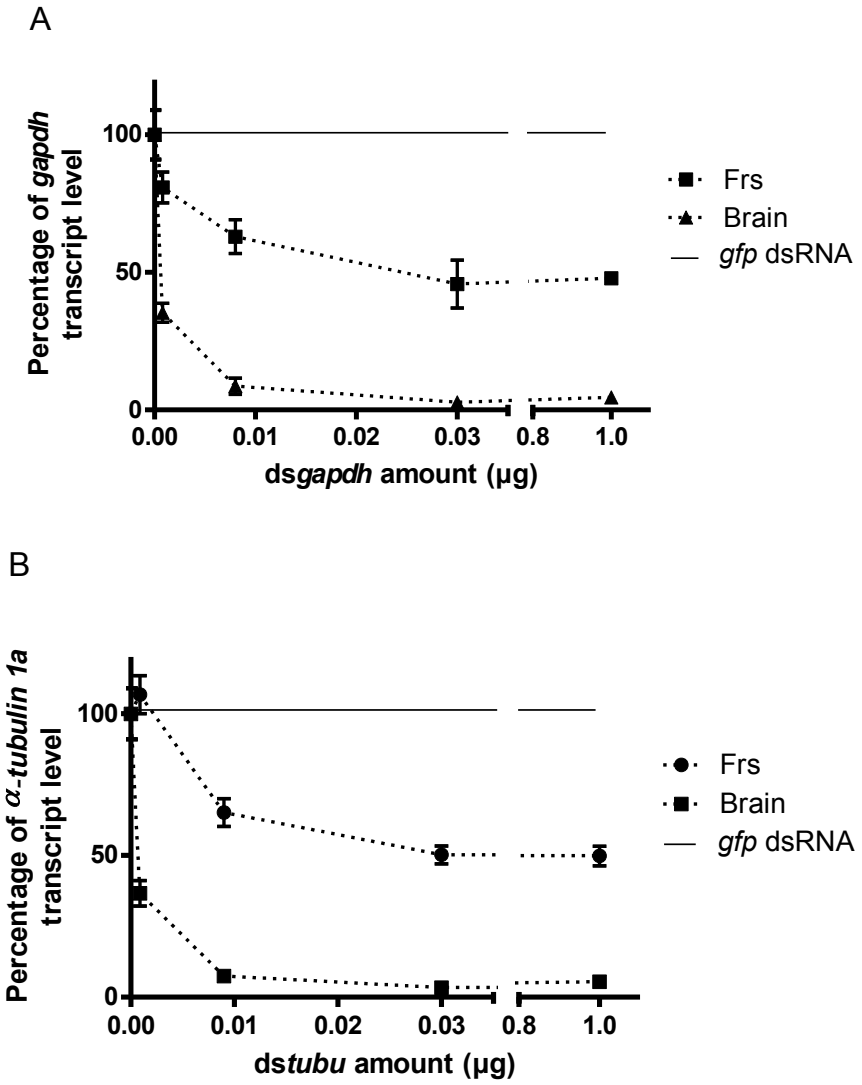


Fig. 2. RNAi-response three days after injection of different quantities of dsRNA for (A) *gapdh* and (B) *alpha-tubulin 1a* (*tubu*) in the female reproductive system (Frs) and brain. The knock down is displayed as the mean percentage of the transcript levels present in the test groups in comparison to the mean relative transcript levels in the control groups that were injected with *gfp* dsRNA. Although the knockdown for the four dsRNA-doses investigated is connected with a broken line, the intermediate dsRNA-doses were not assessed (mean ± SEM, n ≥ 4-6).

Similarly, by measuring the knockdown after three different durations; 1-day, 3-days and 10-days post injection (p.i.), the time-dependency of the RNAi-response was assessed. Five µg *gapdh* dsRNA was applied to saturate the RNAi-machinery, and four different tissues were examined, brain and midgut representing the more responsive tissues and male and female reproductive systems as less robust responders (§ 3.3.1). Our data show that the silencing response increased significantly with time, for all four tissues (Fig. 3A). A severe knockdown was seen 1-day p.i. in the brain and midgut, while this was minimal at this time point in the reproductive tissues. Similar time dependence was observed for *tubu* (Fig. 3B). However, in contrast to silencing the *gapdh* gene, injection of *tubu* dsRNA resulted in locust mortality, with the bulk of the locust population being dead 14-days p.i.. Since initial lethality occurred 7-days after injection of the dsRNA, the latest assessed time point for *tubu* was 6-days, instead of 10-days p.i..

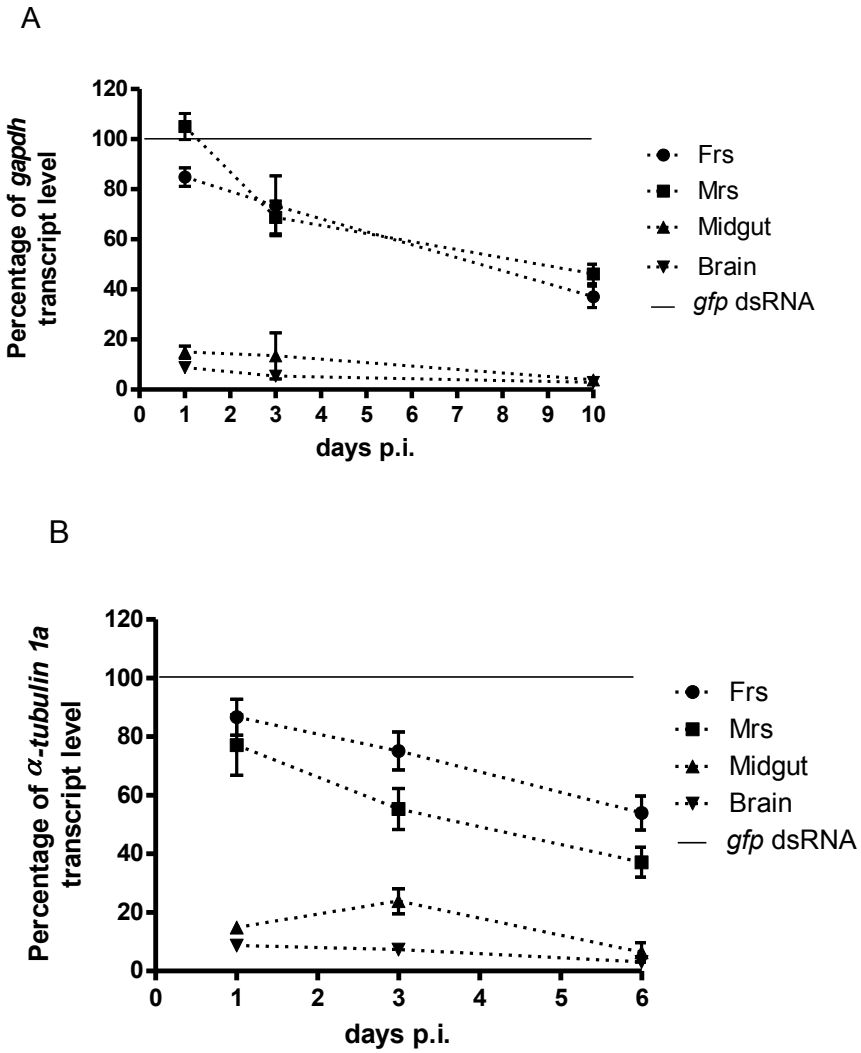


Fig. 3. Time-dependence of the RNAi-response after the injection of 5 µg dsRNA for (A) *gapdh* and (B) *alpha-tubulin 1a* (*tubu*) transcript sequence in the female reproductive system (Frs), male reproductive system (Mrs), brain and midgut. The knock down efficacy is displayed as the mean percentage of the transcript levels present in the test groups in comparison to the mean relative transcript level in the control groups that were injected with *gfp* dsRNA, *i.e.* at each time point (mean ± SEM, n = 6-9). The *gapdh* transcript level decreases in all four tissues in course of time (Frs: $p < 0.0001$, Mrs: $p < 0.0001$, midgut: $p < 0.005$ and brain: $p < 0.0005$). For *tubu* the transcript quantity decreases in the Frs ($p < 0.001$), the Mrs ($p < 0.005$) and the brain ($p < 0.005$). Although the knockdown for the three time points investigated is connected with a broken line, the intermediate time points were not assessed.

The transcript levels of *tubu* and *gapdh* in midgut, brain, female reproductive system and male reproductive system can be found Fig. 4. This demonstrates that the observed differences in RNAi-potency are not correlated to higher different expression levels of these genes in the reproductive systems.

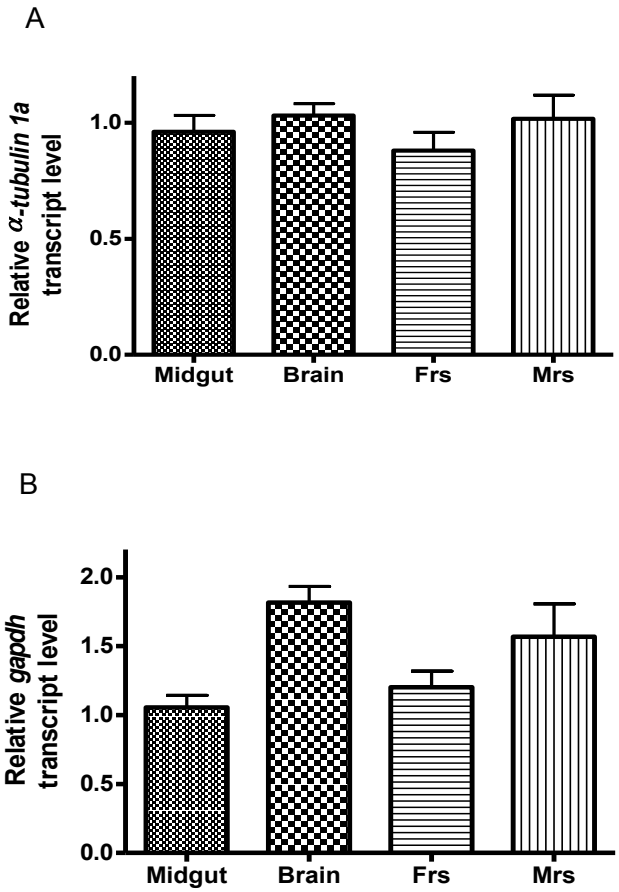


Fig. 4. Four days old adult desert locust were injected with dsRNA of *gfp*. Three days later, the relative gene expression levels of *alpha-tubulin 1a* (*tubu*) and *gapdh* for four tissues; midgut, brain, female reproductive systems (Frs) and male reproductive tissues (Mrs) in *S. gregaria* were determined by means of qPCR (mean ± SEM, n = 8).

3.3.3. Oral delivery of dsRNA

Whereas a single injection of dsRNA is highly effective in mediating RNAi, daily oral delivery of 1 µg of *tubu* dsRNA for 8 times could not trigger RNAi (Fig. 5). We tested two different oral delivery methods (oral injection of dsRNA and administering dsRNA to an artificial feeding medium), targeted two different genes, namely *tubu* and *gapdh*, and tested different doses of dsRNA (up to a single administration of 5 µg

of dsRNA), all without success, while, tracing a blue coloured marker to the midgut lumen approved the oral injection delivery method.

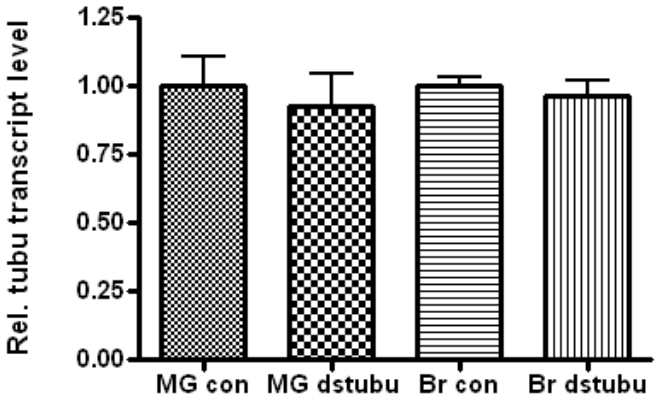


Fig. 5. For 8 times, 1 µg of *tubu* dsRNA was daily orally delivered to the locust. Three days later the *tubu* transcript level was determined by means of qPCR and this for two different tissues, namely the midgut (MG dstubu) and the brain (Br dstubu). As a control, we also determined the *tubu* transcript level in locust that received, each time, the same volume of *Sg*-Ringer solution (MG con and Br con) (mean ± SEM, n = 5).

3.4. Discussion

In this chapter, we report on a detailed examination of the sysRNAi-response in the desert locust, *S. gregaria.* Our results demonstrate that, in contrast to the relatively high resistance against sysRNAi in many dipteran and lepidopteran species (Miller *et al.*, 2008; Belles, 2010; Terenius *et al.*, 2011), injection of as little as 15 pg of dsRNA per mg tissue is sufficient to almost completely silence specific gene expression in adult desert locusts (Fig. 2). This finding is in accordance with the recently published highly sensitive RNAi-response in the migratory locust, *L. migratoria*, where it was reported that injection of 30 pg of dsRNA per mg tissue could generate potent silencing effects in adult *Locusta* (Luo *et al.*, 2012) and in the red flour beetle, *T. castaneum,* that displayed detectable RNAi-effects upon injection of less than 0.1 ng of dsRNA per larvae (Miller *et al.*, 2012). In addition, we show that the reduction in transcript level persists for a long time, with an increase in knockdown efficacy during (at least) a ten-day period (Fig. 3), thereby rendering the desert locust an extremely RNAi-sensitive and -persistent insect species.

Whereas in nematodes, plants and fungi the high sensitivity and persistency of RNAi is attributed to the presence of RdRPs that amplify the dsRNA, in the genome of insects there are no canonical *RdRP-like* sequences present (Tijsterman *et al.*, 2002; Baulcombe, 2007). Whether other dsRNA-amplification mechanisms exist in insects, as suggested by Lipardi and Paterson (2009), or alternative mechanisms are used to achieve this effective systemic silencing process is still to be determined. In the absence of dsRNA-amplification, it would be expected that the RNA induced silencing complex (RISC) is a multiple turnover enzyme with a long cellular half-life. The highly effective sysRNAi-response, in combination with the available transcript sequence information of *S. gregaria* (§ 2.2 and 2.3), makes the desert locust an attractive organism for 'loss of function' analyses, which could lead to the development of new insect control methods.

However, a more moderate RNAi-response was observed in the adult male and female reproductive systems (Fig. 1). This effect was most pronounced shortly after injection of dsRNA, while eventually also these tissues displayed a severe knockdown (Fig. 3). It is notable that the dsRNA-dose dependence was highly similar to this in the more responsive brain (Fig. 2). This reduced RNAi-susceptibility is in contrast to the tissue-dependent response in *D. melanogaster*, where several tissues seem to be completely refractory towards sysRNAi (Miller *et al.*, 2008). The tissue-dependency of the sysRNAi-response will be further assessed in Chapter 4.

Targeting the *tubu* mRNA gene product resulted in mortality in the locusts' population (section 3.2), confirming knockdown at the protein level and suggesting that RNAi may contribute to promising strategies for selectively controlling locust pests. Yet, cellular exposure to dsRNA and off-target effects of the siRNAs might have contributed to the observed mortality in the locust population.

For RNAi-based insect pest management, oral delivery of dsRNA via *in planta* or *in bacteria* produced dsRNA could be a method of choice. However, the desert locust displays reduced sensitivity upon oral delivery of dsRNA. Moreover, we remained unable to generate gene silencing effects via oral delivery of dsRNA (Fig. 5). These experimental data is in accordance to the reports made for *L. migratoria*, where oral delivery of high dsRNA quantities did not result in observable gene silencing effects (Luo *et al.*, 2013). In contrast, many other insect species were shown sensitive towards oral delivery of dsRNA (Baum *et al.*, 2007; Mao *et al.*, 2007). Yet, in general,

oral delivery of dsRNA requires higher dsRNA-doses than intra-abdominal injection (Terenius *et al.*, 2011). In this context, the identification of factors affecting the RNAi-susceptibility is of major interest and is further discussed in Chapter 5.

Taken together, our data identify the desert locust as a highly interesting model to study regulatory mechanisms of RNAi in insects. In particular, the desert locust displays a highly robust and sensitive RNAi-response upon injection of dsRNA, while the reproductive tissues displayed a tissue-dependent reduction of RNAi-potency and different sensitivity towards intra-abdominal injection and oral delivery of dsRNA.

Chapter 4

Tissue-dependence of the systemic RNAi-response in *Schistocerca gregaria*

This chapter is partially based on a paper published in Insect Biochemistry and Molecular Biology (Wynant *et al.*, 2012).

Chapter 4: Tissue dependence of the systemic RNAi-response in *Schistocerca gregaria*

4.1. Introduction

RNA interference (RNAi) has transformed insect science, since it enables the suppression of a gene of interest (Belles, 2010). In addition, RNAi has potential to contribute to the specific control of agricultural pest insects and protect insects againsts viral infections.

Upon cell entry, dsRNA is cleaved by the RNase III endonuclease Dicer (Dcr) into small dsRNA-fragments of approximately 20-25 bp. These fragments are subsequently loaded into the RNA induced silencing complex (RISC) that will unwind the siRNA and use the antisense strand as a guide to find complementary mRNA sequences in the cell. These mRNAs will be cleaved by Argonaute (Ago), which is the catalytic component of RISC. Dcr enzymes produce two groups of small dsRNAs, (i) micro (mi)RNA, which are processed from endogenous gene transcripts and function in the regulation of physiological processes as development, differentiation and metabolism, and (ii) short interferening (si)RNAs that are generated from long dsRNA-fragments derived from viruses or transposable elements. Experimental use of RNAi by administering long dsRNA-fragments exploits the siRNA-pathway (Hammond, 2005).

Insects possess two Dcr-enzymes (Dcr1 and Dcr2), which typically generate miRNAs and siRNAs, respectively, as demonstrated in *D. melanogaster* and *T. castaneum* (Tomoyasu *et al.*, 2008). In addition, studies have shown that Ago2 is the main Ago-protein involved in siRNA-directed mRNA cleavage, while Ago1 guides miRNA-directed gene suppression (Okamura *et al.*, 2004; Siomi *et al.*, 2004; Forstemann *et al.*, 2007). However, the biogenesis of small RNA duplexes is uncoupled from their loading into Ago1 or Ago2, but is governed by the structure of the duplex. Duplexes that contain bulks and mismatches are sorted into Ago1, while duplexes with a greater double stranded structure will be sorted into Ago2. However, since increasing the Dcr2/R2D2 complex concentrations reduces the quantity of siRNAs loaded into Ago1, it was demonstrated that sorting can create competition for the substrate (Forstemann *et al.*, 2007; Tomari *et al.*, 2007; Ghildiyal and Zamore, 2009).

Nonetheless, delivery of dsRNA into the cells remains challenging. Whereas, in nematodes and several insect species injection of dsRNA into the body cavity and, in some cases, even oral delivery of dsRNA results in gene silencing throughout the body, a phenomenon that is known as systemic (sys)RNAi, several mammalian and insect species are largely refractory towards injected dsRNA (Belles, 2010). For an organism to display a sysRNAi-response, individual cells must be able to take up dsRNA from their environment. At state, two dsRNA-uptake mechanisms have been identified in invertebrates: transmembrane channel mediated transport, exemplified by the SID1 transporter of the nematode *C. elegans* (Winston *et al.*, 2002), and receptor-mediated endocytosis, which was proven to direct dsRNA-uptake in *D. melanogaster* S2 cells (Saleh *et al.*, 2006; Ulvila *et al.*, 2006).

Yet, once in the cell, dsRNA-degrading enzymes, such as the exoribonuclease 1 (ERI1), may negatively regulate the silencing process (Kennedy *et al.*, 2004). ERI1 is an intracellular dsRNAse that cleaves dsRNA-molecules and is correlated to negatively regulate the RNAi-response in the nematode, *C. elegans* (Boisson *et al.*, 2006). In the fission yeast *Saccharomyces pombe*, loss of ERI1 causes increased levels of small interfering RNAs (siRNAs) corresponding to centromeric repeats and a concomitant increase in RNAi-dependent heterochromatin formation at these genomic loci (Iida *et al.*, 2006). Analysis of ERI1 in *C. elegans*, humans and fission yeast has demonstrated its siRNA-degradation activity *in vitro* (Dominski *et al.*, 2003; Kennedy *et al.*, 2004; Iida *et al.*, 2006; Yang *et al.*, 2006). On the other hand, *eri1* mutant flies did not have markedly enhanced RNAi-responses (Kupsco *et al.*, 2006). Therefore, it remains uncertain whether Eri1 can also affect the RNAi-response in insects.

In the previous chapter, we demonstrated that the desert locust, *S. gregaria,* displays a highly efficient sysRNAi-response. However, silencing genes in the adult male and female reproductive tissues was less effective than in the other tissues investigated. Here, we aim to identify mediating factors for the tissue-dependency of RNAi in the desert locust. Therefore, the tissue transcript profile of RNAi-components that are involved in (i) the cell-autonomous core RNAi-machinery (*dcr2* and *ago2*), (ii) dsRNA-uptake (*sid1 like*) and (iii) degradation of intracellular dsRNA levels (*eri1*) was determined by means of quantitative real time (q)PCR.

4.2. Materials and Methods

4.2.1. Sequence information in the *S. gregraria* EST-database

Transcript sequence information of *S. gregaria glyceraldehyde phosphate dehydrogenase* (*gapdh*), *alpha-tubulin 1a* (*tubu*), *dicer-2* (*dcr2*), *argonaute-2* (*ago2*), *exonuclease 1* (*eri1*) was retrieved from the annotated *S. gregraria* EST-database (Badisco *et al.*, 2011a). There was no sequence available for the *sg-sid1* transcript. However, based on the orthologous sequence found in *S. americana* by Dong and Friedrich (2005), a fragment of 435bp was amplified. The DNA fragments were cloned into the pCR®4-TOPO® vector by means of the TOPO TA Cloning® Kit for Sequencing (Invitrogen). The sequences of the inserted DNA fragments were determined using the ABI PRISM BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems). The obtained sequences were subsequently compared with BLAST to NCBI nucleotide databases of other insects and *in silico* translated into the corresponding amino acid sequences. The latter were then aligned against orthologous sequences of other insects (ClustalW), and the predicted protein domains were identified (NCBI, CD-search). In addition, since Dcr1 and Ago1 are related to Dcr2 and Ago2 proteins, respectively, we have constructed an unrooted phylogenetic tree (ExPASy, ETH Zurich) to determine the correct identity of the sequences retrieved from *S. gregaria*.

4.2.2. Rapid amplification of cDNA ends

In order to obtain the complete ORF of the *sid1 like* transcript sequence of *S. gregaria*, a Rapid Amplification of cDNA Ends (RACE) protocol was performed, following the instructions of the SMARTer™ RACE cDNA Amplification kit (Clontech Laboratories Co.). Adapter primers were included in the kit, while gene-specific primers were derived from the original PCR fragment:

5' RACE: CACCAGCCATGCCAATCAAGATGAC
nested 5' RACE: AATAAGCACTAGCATTTATGTCTGGGTG
3' RACE: TGGCTGGTGTGCTAAAAGGCGGAGTG
nested 3' RACE: TTTGATGTCCGTCCGTGCCAGTCCACTGC

The amplified fragments were analyzed with 1% agarose gel electrophoresis and purified using the GenElute gel extraction kit (Sigma-Aldrich co.). The fragments were subsequently cloned in a pCR®4-TOPO® vector using the TOPO TA Cloning® Kit for Sequencing (Life Technologies Co.) and sequenced according to the ABI PRISM BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems).

4.2.3. Synthesis of dsRNA

Double stranded RNA for *gapdh* (447 bp), *tubu* (545 bp), *green fluorescent protein* (*gfp*, 589 bp), *dcr2* (330 bp) and *ago2* (461 bp) were synthesized by using the MEGAscript RNAi kit (Ambion) as described in § 2.4.

4.2.4. RNA-extraction and cDNA-synthesis

The RNA of individual tissues was subsequently extracted. The Lipid tissue extraction kit (Qiagen) was utilized to extract RNA from the samples. This was performed in accordance to the methods described in § 2.7. The RNA-samples were stored at -80°C. Next, equal quantities of RNA were used as template to produce cDNA. The cDNA synthesis was performed using the Superscript III reverse transcriptase kit (Invitrogen), random hexamer primers (Invitrogen) and dNTPs (Roche diagnostics GmbH) as described in § 2.7.

4.2.5. Quantitative real time-PCR

The knockdown was assessed by means of qPCR in accordance to the described procedures in § 2.8 and the data were analysed by non-parametric statistics in GraphPad prism 5 (GraphPad).

4.3. Results

4.3.1. Retrieval and evaluation of *argo2*, *dcr2* and *eri1* transcript sequences from the *S. gregaria* EST database

In the available *S. gregaria* EST-database, we found partial transcript sequences of *dcr2*, *ago2* and *eri1* by means of reciprocal tBLASTn. To confirm their correct identity, the predicted protein domains in the deduced protein sequences were determined.

The partial transcript sequence of *dcr2* was predicted to encode two RNase III domains, while a DUF1785-, PAZ- and PIWI-domain were found in the deduced Ago2 sequence and the partial transcript sequence of *eri1* was predicted to encode for a DEDDh 3'-5 exonuclease domain (Fig. 1).

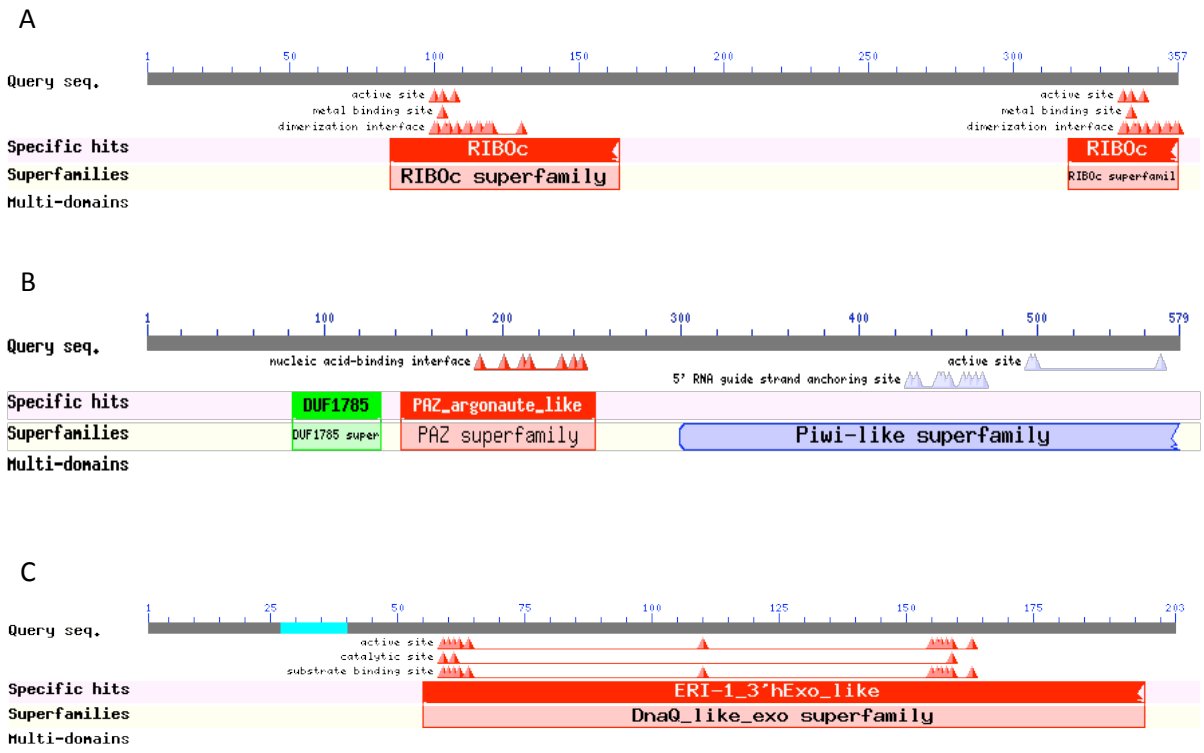


Fig. 1. The protein domains that are present in the (available) deduced amino acid sequences of (A) *dicer-2* (*dcr2*), (B) *argonaute-2* (*ago2*) and (C) *exoribonuclease* (*eri1*), predicted with CD-search (NCBI). In addition, nucleic acid and metal binding sites, as well as dimerization sites are indicated with coloured triangles.

However, due to high homology between Dcr1 and Dcr2, and Ago1 and Ago2 their correct identity was further validated by the construction of an unrooted phylogenetic tree. Since the fragments clustered with Dcr2 and Ago2 sequences of other insect species, including these of *T. castaneum* and *D. melanogaster*, whose function in siRNA-directed RNAi were experimentally determined (Lee *et al.*, 2004; Tomoyasu *et al.*, 2008; Czech *et al.*, 2009), we confirmed that these transcript sequences are indeed the *dcr2* and *ago2* homologues of the desert locust (Fig. 2).

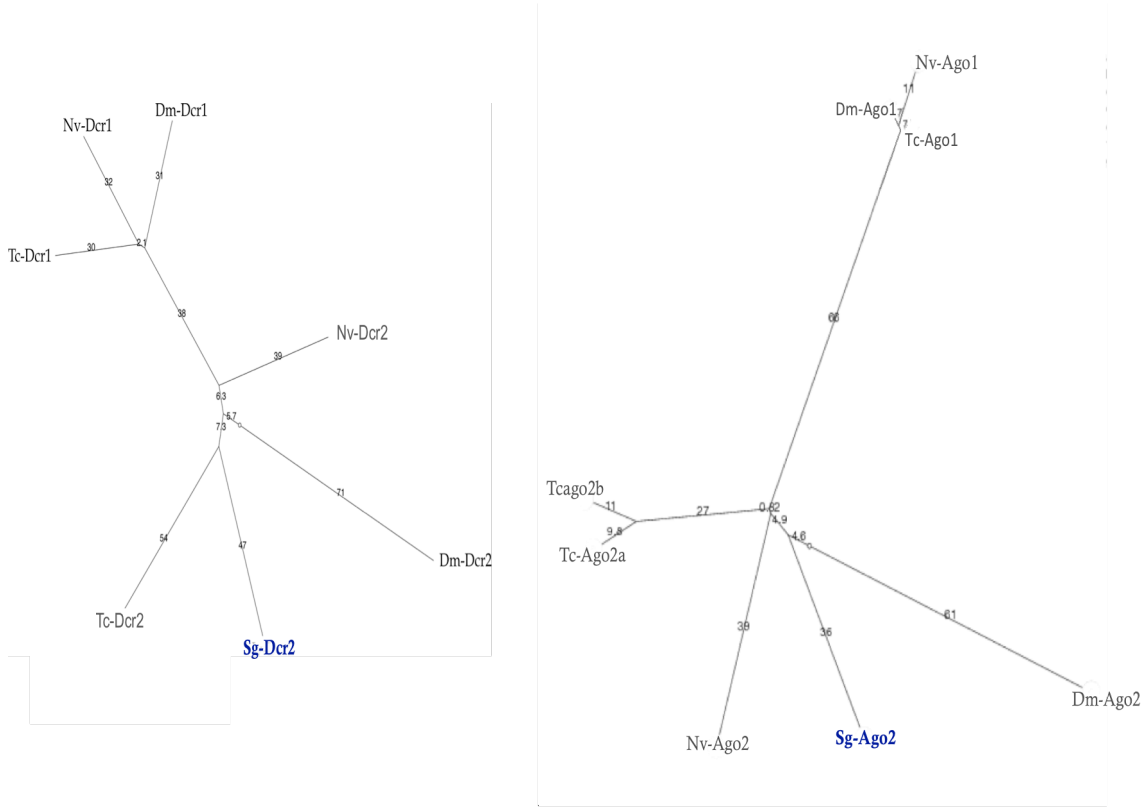


Fig. 2. Unrooted phylogenetic tree (Expasy, ETH Zurich) with the available Dicer (Dcr, left) or Argonaute (Ago, right) amino acid sequences of *Schistocerca gregaria*, *Drosophila melanogaster*, *Tribolium castaneum* and *Nasonia vitripennis* included in the data set.

4.3.2. Amplification and evaluation of the *sid1 like* sequence

Since no sequence of the *Sg-sid1 like* transcript was readily available, we designed degenerated primers and succeeded in amplifying a small fragment of *ca.* 300 bp. Next, by using RACE, we obtained a sequence with an ORF of 2364 bp that was highly similar to *C. elegans* SID1, both containing a signal peptide followed by 11 putative transmembrane regions (determined using Phobius software) (Fig. 3). The amplified *Sg-sid1* sequence was, later on, confirmed by the sequence present in the available *S. gregaria* transcriptome database.

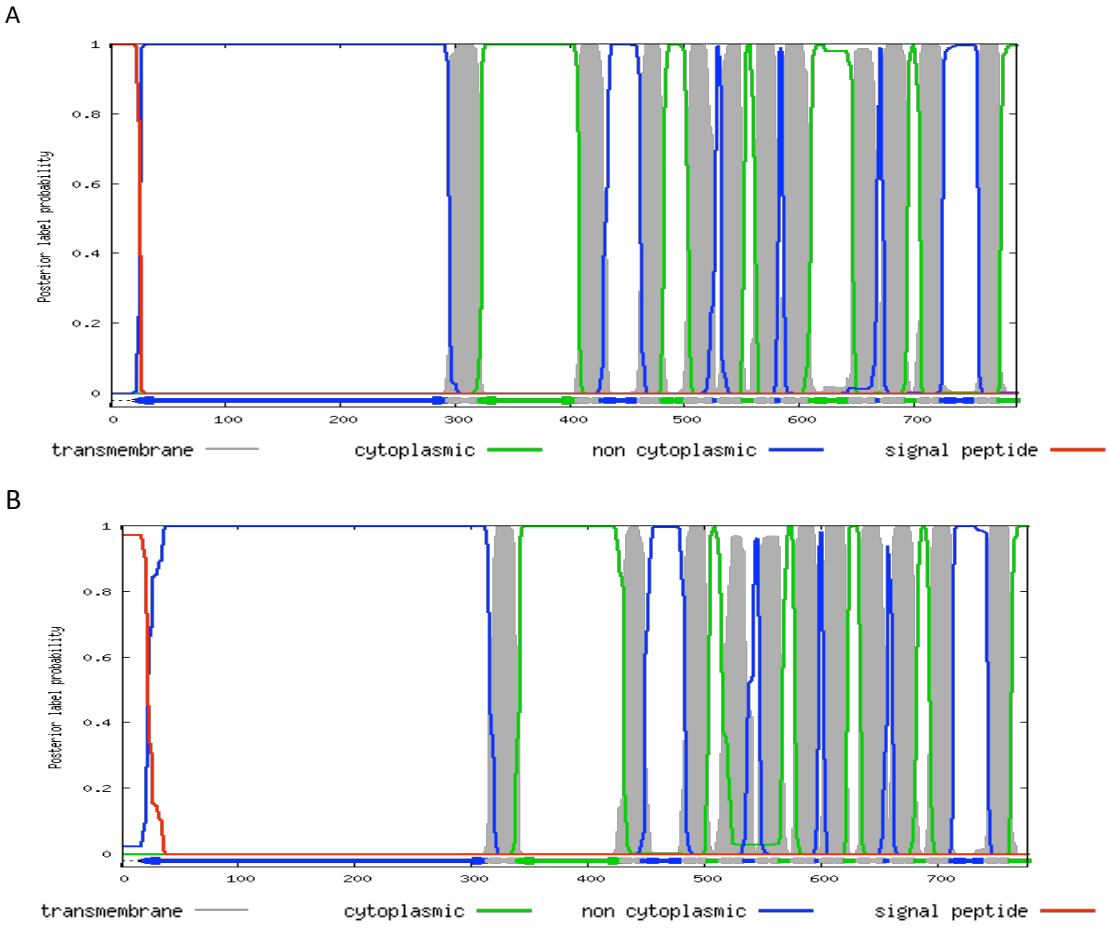


Fig. 3. The predicted topological structure of (A) SID1 like of *S. gregaria* and (B) SID1 of *C. elegans* using Phobius software.

4.3.3. The reproductive system differentially expresses *ago2* and *dcr2* transcript levels

We measured the tissue distribution of *dcr2*, *ago2*, *sid1 like* and *eri1* transcript levels in adult *S. gregaria*. Our results (Fig. 4) showed that the transcript levels of *dcr2* were relatively lower in female and higher in male reproductive organs, while significantly lower *ago2* expression levels were observed in the male reproductive system. On the other hand, the transcript levels of *sid1* and *eri1* remained indistinguishable between the different tissues investigated. Similar results were obtained for four and ten days old adult locusts.

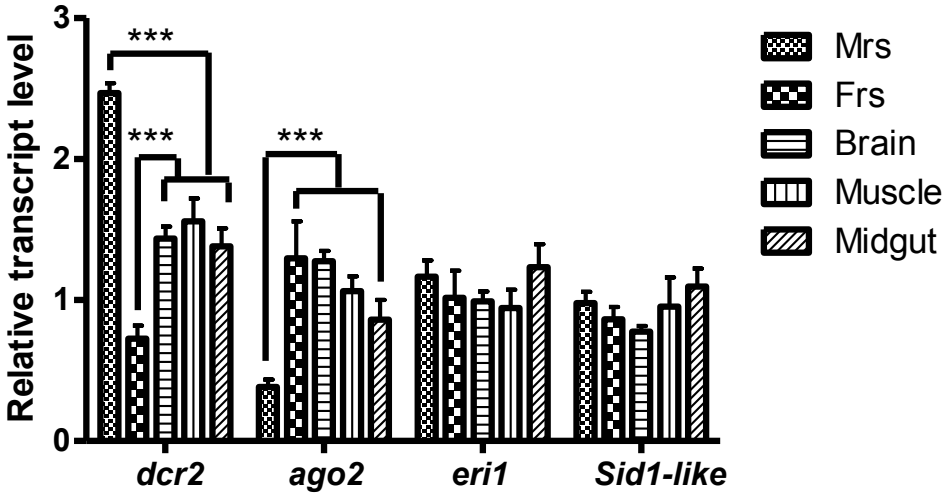


Fig. 4. Tissue-dependent transcript level profile of several putative RNAi-genes. The relative transcript levels of *dcr2* are higher in the male reproductive system (Mrs) and reduced in the female reproductive system (Frs), while *ago2* transcript levels are reduced in the Mrs. Each bar represents the mean of six independent pools of (four and ten days old) male and female adults (40, 40, 10, 10, 10 and 10 animals/pool) (mean ± SEM, ***: $p < 0.0005$).

4.3.4. The ovaries are the less responsive part of the female reproductive system and have lower *dcr2* transcript levels

The female reproductive system was further subdivided into ovaries and oviducts. Measuring the sysRNAi-susceptibility in these tissues revealed a very strong knockdown in the oviducts, while a significantly lower knockdown was obtained in the ovaries (Fig. 5a). Next, we tested if the reduced *dcr2* expression level in the female reproductive system is also limited to the ovaries. The expression levels of *dcr2* and *ago2* were measured, and as displayed in Fig. 5b, the ovaries contain relatively less *dcr2* transcript levels, while no significant differences could be reported for *ago2*.

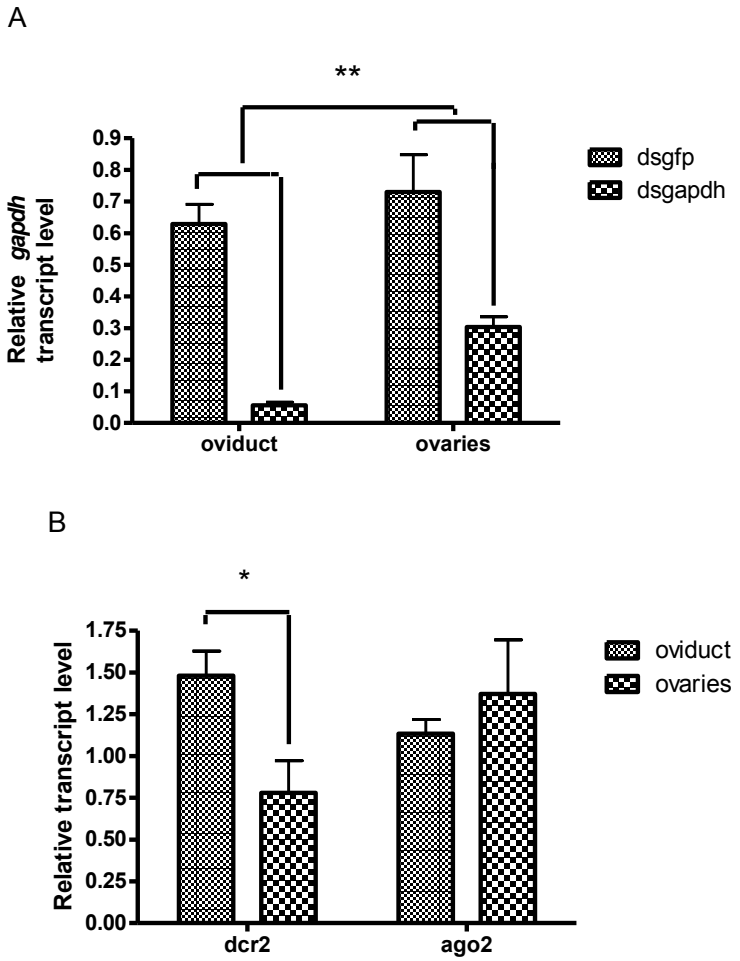


Fig. 5. Reduced RNAi-susceptibility and lower transcript levels of *dcr2* in the ovaries in comparison to the oviducts. (A) Knock down of *gapdh* in oviducts and ovaries (mean ± SEM, n = 6, **: $p < 0.01$). (B) Relative transcript levels of *dcr2* and *ago2* in the oviducts and ovaries (mean ± SEM, n = 5-6, *: $p < 0.05$).

4.3.5. Lower *dcr2* or *ago2* transcript levels result in reduced RNAi-susceptibility

In order to investigate whether the expression levels of either *dcr2* or *ago2* can influence the RNAi-susceptibility, we silenced these components by injecting 100 ng of gene-specific dsRNA (a quantity that should be sufficient to saturate the RNAi-machinery (Chapter 3)). As a control, 100 ng of *gfp* dsRNA was injected. Six days later, a second injection was performed with 100 ng of *tubu* dsRNA. To be able to determine the knockdown effectiveness, a second control group was taken into account that was treated twice with a *gfp* dsRNA injection. With preliminary results, we already observed potent knockdown effects fourteen hours after the injection of *tubu* dsRNA.

Therefore, the efficiency of the RNAi-response was assessed 14 hours p.i.. The data in Fig. 6A show a successful knockdown of *ago2* and *dcr2* in midgut tissue and, as demonstrated in Fig. 6B, this resulted in a reduced knockdown of the *tubu* gene expression in the midgut tissue.

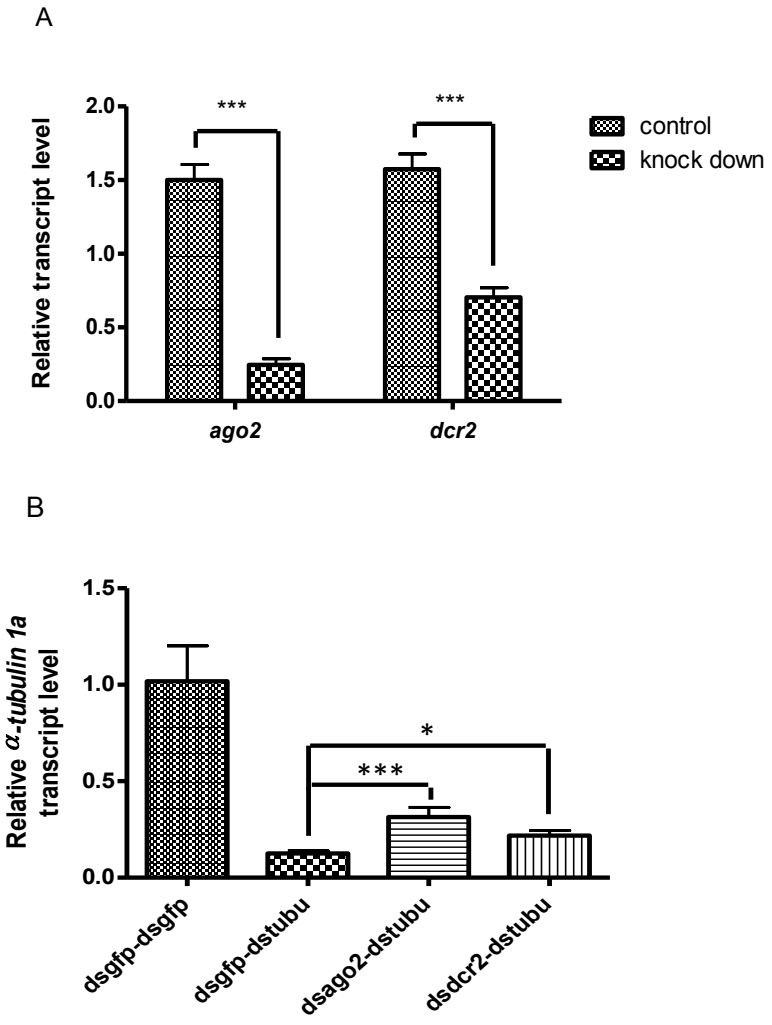


Fig. 6. Both *ago2* and *dcr2* are involved in the RNAi-pathway. (A) An effective knockdown of *ago2* and *dcr2* transcript level was obtained (mean ± SEM, n = 6-10; ***: $p < 0.001$). (B) The locusts were injected with either 100 ng of ds*gfp*, ds*ago2* or ds*dcr2* and six days later followed by a second dsRNA injection of 100 ng of *alpha-tubulin 1a* (ds*tubu*), indicated as respectively ds*gfp*-ds*tubu*, ds*ago2*-ds*tubu* and ds*dcr2*-dst*ubu*. In addition, a second control group that was twice injected with ds*gfp*, indicated as ds*gfp*-ds*gfp*, was also assessed. Fourteen hours after the second injection midgut tissue was microdissected and the relative transcript level was then measured with real-time qPCR. Statistical analysis was performed between the ds*ago2*-ds*tubu* or ds*dcr2*-ds*tubu* group and the group ds*gfp*-ds*tubu* (mean ± SEM, n = 7-10, ***: $p < 0.001$ and *: $p < 0.05$).

Yet, it remains possible that silencing *dcr2* or *ago2* directly influences the *tubu* gene expression level, rather than the RNAi-susceptibility. Therefore, the *tubu* expression level was also determined 6 days after injection of *dcr2* or *ago2* dsRNA, without injection of *tubu* dsRNA. As demonstrated in Fig. 7, no differential *tubu* transcript level was reported.

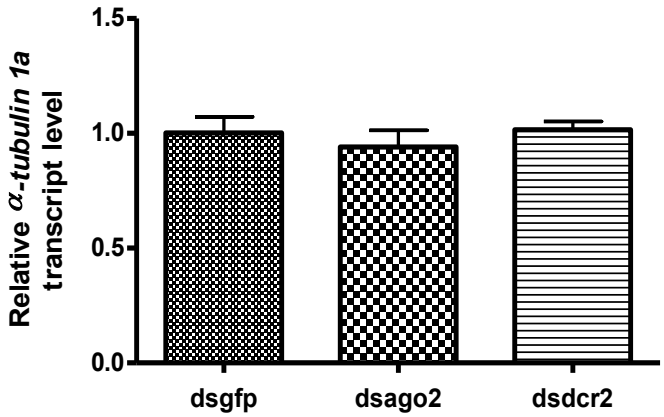


Fig. 7. Silencing of *ago2* or *dcr2* transcript levels had no direct effect on the *alpha-tubulin 1a* transcript level in the midgut. The expression level was determined 6 days after injection of ds*gfp*, ds*ago2* or ds*dcr2* (mean ± SEM, n = 5).

4.4. Discussion

Regarding the reduced RNAi-potency in the reproductive system of the desert locust (chapter 3), we sought to investigate the possible role of (i) the core RNAi-machinery, (ii) dsRNA-transport from the hemolymph into the tissue and (iii) dsRNA-degradation in the tissues. Therefore, we determined the transcript profile of RNAi-components that are involved in these three processes and demonstrated that the *dcr2* and *ago2* transcript level is lower in the adult female and male reproductive systems, respectively. Yet, their functioning in RNAi in locusts was still unclear. So, we silenced *dcr2* and *ago2* and confirmed that reduced expression levels of these genes can indeed affect the RNAi-potency in the desert locust, which is in agreement with their role in *D. melanogaster* and *T. castaneum* (Lee *et al.*, 2004; Tomoyasu *et al.*, 2008; Czech *et al.*, 2009). Nevertheless, this is the first report on their function in a member of the Orthoptera. These components clearly play a crucial role in the core

RNAi-pathway of *D. melanogaster* (Kim *et al.*, 2006). However, silencing *dcr2* or *ago2* in the desert locust did not completely abolish the RNAi-response (Fig. 6). This should not necessarily imply that their involvement in RNAi is less important in *S. gregaria*. First, RNAi only generates a silencing effect at the post-transcriptional level. Second, previous studies have demonstrated that the protein turnover rate can be a determinate factor for the potency of the knockdown at the (functional) protein level (Ott *et al.*, 2012). A slow protein turnover rate may thus account for a rather moderate functional effect of the RNAi-response.

Although both male and female adult reproductive systems display less potent knockdown effects, their reported transcript profiles for the RNAi-genes *ago2* and *dcr2* are dissimilar. Whereas *dcr2* transcript levels are lower in the female reproductive system, the male reproductive system contains less *ago2* and more *dcr2* transcripts (Fig. 4). This finding illustrates the complexity of RNAi and indicates that the process may be regulated differently in different tissues. The reasons for this are currently not clear. One possible explanation could reside in the fact that, as reported in *D. melanogaster*, some cross-talk and overlap may exist between the siRNA- and miRNA-pathways (Lee *et al.*, 2004; Zhou *et al.*, 2008a). Whereas the siRNA-pathway is primarily an anti-viral immune response that recognizes dsRNA-molecules, the miRNA-pathway regulates the expression of endogenously produced mRNA-targets (Carthew and Sontheimer, 2009). This could mean that regulation of the expression level of components of the siRNA-pathway might also affect the miRNA-pathway, or *vice versa,* and that compensatory control mechanisms might be required. An analysis of the components of the miRNA-pathway would therefore be of interest to verify this hypothesis. Unfortunately, sequence information of these components was either not available in the *S. gregaria* EST-database, or the fragments were too short to design suitable qRT-PCR primers. Yet, the recently available *S. gregaria* transcriptome database contains sequence information for several additional components of the siRNA-, miRNA- and piRNA-pathway. Therefore, future perspectives could reside in determining the transcript profile of these components and testing their involvement in the siRNA- or miRNA-directed pathways. This might be off particular interest, since studies have demonstrated that Ago3 can also be involved in siRNA-directed silencing (Hoa *et al.*, 2003; Kolliopoulou and Swevers, 2013). In this respect, these Agos could (partially) compensate for the loss of Ago2.

In addition, dsRNA can be unwound by editing enzymes in ovarian tissue of silkmoths (Skeiky and Iatrou, 1991). Therefore, in this way RNAi might also be restricted in these tissues, making *dcr-2* not absolutely necessary. In this context, lower Dcr2 levels might seem logical. In any case, this would still result in reduced sensitivity of the cell-autonomous RNAi-machinery.

Nevertheless, it remains remarkable that specifically in the reproductive system a more moderate RNAi-potency is observed and that this coincides with differential transcript levels of *dcr2* or *ago2*. Since silencing either *dcr2* or *ago2* is sufficient to reduce the knockdown efficiency (Fig. 6), reduced transcript levels of one of these components may account for a lower RNAi-potency. It is therefore probable that each of these enzymes represents a rate-limiting step for the RNAi-process. However, it remains unclear to what extent an increase of *dcr2* transcripts (as observed in the male reproductive system) could (partially) undo the effects caused by lower *ago2* transcript levels.

Interestingly, low transcript levels of the core RNAi-components, *Bm-R2D2*, *Ms-dcr2* and *Ms-ago2,* have previously been considered as a possible explanation for the apparent inefficacy of RNAi in *B. mori* and *M. sexta*, respectively (Swevers *et al.*, 2011; Garbutt and Reynolds, 2012). In addition, in *A. gambiae*, it was shown that the less responsive salivary glands displayed lower *dicer* and *argonaute* transcript levels (Boisson *et al.*, 2006). On the other hand, in *C. elegans*, the relative inefficiency of neuronal RNAi appeared mainly due to the low expression levels of the *Ce-sid1* dsRNA-transporter and high expression levels of the *Ce-eri1* dsRNAse (Kennedy *et al.*, 2004; Calixto *et al.*, 2010). In the present study, we have assessed the transcript levels of *sid1* and *eri1* in various tissues of the desert locust, but no tissue-dependent differences were observed (Fig. 4).

In conclusion, in this chapter we have identified two components of the RNAi-process (Dcr2 and Ago2) and correlated their transcript level to the tissue-dependent RNAi-potency in the desert locust. Therefore, a possible explanation for the reduced RNAi-potency in the adult reproductive tissues might (partially) reside in lower transcript levels of components of the core RNAi-machinery.

Chapter 5

Regulation of systemic RNAi in *Schistocerca gregaria*: dsRNases and lipophorins

Chapter 5: Regulation of systemic RNAi in *Schistocerca gregaria*: dsRNases and lipophorins

5.1. Introduction

RNA interference (RNAi) is a mechanism of post-transcriptional gene silencing triggered by double stranded (ds)RNA molecules. Under natural conditions, RNAi is triggered by dsRNA structures that are produced during the replication cycle of viruses or generated from repetitive elements and transposons in the cellular genome (Hammond, 2005). Thanks to its robustness and specificity, RNAi has become a widely used method to silence genes in many eukaryotic systems and may contribute to novel strategies to control agricultural pests, including a number of insect species.

In chapter 3, we showed that abdominal injection of dsRNA into the body cavity is a highly effective delivery method for gene silencing in the desert locust. Moreover, targeting the housekeeping gene *alpha-tubulin 1a* (*tubu*) generated mortality in the locust population. Yet, for in field applications of RNAi-based pest control, oral delivery of the dsRNA would be of particular interest. For this purpose, one could use transgenic plants or micro-organisms that express dsRNAs targeting vital insect genes. However, the RNAi-response of the desert locust does not respond well towards orally administrated dsRNA (Chapter 3).

Arimatsu *et al.* (2007) have purified an alkaline nuclease that is secreted into the midgut of the silkmoth, *B. mori*, where it can digest dsRNA. This nuclease is characterized by the presence of a single DNA/RNA non-specific nuclease (NN)-domain that is proceeded by a spacer region and a signal peptide. Expression of this protein has been detected in the middle and posterior midgut of larvae, whereas no expression was detected in the anterior midgut, silk glands, fat body and malpighian tubules (Arimatsu *et al.*, 2007). Recently, Garbutt *et al.* (2013) reported on dsRNA-degradation activity in serum of the tobacco hornworm, *M. sexta*, and the german cockroach, *Blatella germanica,* and showed more rapid degradation of the dsRNA in the serum of *M. sexta*. The knowledge that *M. sexta* is poorly RNAi-sensitive towards injection of dsRNA, while *B. germanica* is characterized by a highly potent systemic (sys)RNAi-response, prompted the authors to suggest that the persistence of dsRNA in the hemolymph might be a determinant factor for the susceptibility of insect

species to RNAi (Garbutt *et al.*, 2013). Therefore, it is plausible to consider that dsRNA-degradation in the alimentary tract of the desert locust might contribute to the deficiency of the RNAi-response upon feeding dsRNA.

In the hemolymph of the silkmoth, *B. mori*, lipophorins can also bind to dsRNA (Sakashita *et al.,* 2009). Moreover, although lipophorins are typically known for their ability to transport dietary lipids in insects, recent studies have illustrated that lipophorins also play an essential role in anti-bacterial (Gotz *et al.*, 1997; Dettloff *et al.*, 2001; Cheon *et al.*, 2006; Ma *et al.*, 2006) and anti-fungal immunity (Whitten *et al.*, 2004).

In this chapter, data are presented concerning dsRNA-binding proteins in serum and midgut juice of the desert locust, with the aim to identify contributing factors for the distinct RNAi-success upon oral delivery and injection of dsRNA.

5.2. Materials and Methods

5.2.1. Retrieval of transcript sequence information from the *S. gregaria* transcriptome database

Transcript sequence information for *dsRNase1*, *dsRNase2*, *dsRNase3*, *dsRNase4*, *endonuclease G* (*endoG*), *apolipophorin 1/2* (*apo1/2*) and *apolipophorin 3* (*apo3*) was retrieved from the *S. gregaria* transcriptome database with reciprocal tBLASTn (NCBI). The deduced amino acid sequences, determined by *in silico* translation using Prosite (ExPASy, ETH Zurich), were used to predict the protein domains present (Pfam, Sanger institute) and the presence of a signal peptide sequence (SignalP 4.1, CBS). To validate the transcript sequence information, the cDNA fragments were cloned into the pCR®4-TOPO® vector by means of the TOPO TA Cloning® Kit for Sequencing (Life technologies). The sequences of the inserted DNA fragments were determined using the ABI PRISM BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems).

5.2.2. Phylogenetic analysis

Nucleotide sequence information for *dsRNase* and *endoG* transcripts was retrieved from Genbank (NCBI) and *in silico* translated into the corresponding amino acid sequence (Prosite, ExPASy). The identity of these fragments was confirmed by

reciprocal tBLASTn (NCBI) and by verifying the presence of an NN-domain (Pfam, Sanger institute). Next, the amino acid sequences of the NN-domains were compared with T-coffee alignment software using the BLOSUM matrix (EMBL-EBI). The region with highest homology, which was approximately 100 amino acids in length, was selected with Jalview sofware. The latter was used for the construction of a phylogenetic tree using the maximum likelihood method with 100 bootstraps (PhyML). Different substitution models were assessed; including models based on the LG, Dayhoff, Jones-Taylor-Thornton, Blosum62 and HIVb matrices. In addition, we assessed the Muscle alignment software (MEGA5.1). In all cases, phylogenetic trees with similar structures were obtained.

5.2.3. Collection of hemolymph and midgut juice

After dissecting one thoracic leg of an adult desert locust, hemolymph of adult locusts was collected with capillary tubes. *S. gregaria* serum was prepared by leaving the hemolymph for 1 minute at room temperature. Next, the clot was removed by centrifuging at 3,000 x rpm for 5 minutes. The resulting supernatants (serum) was collected in a new tube and stored at -20°C.

Adult locusts were starved for half a day. From these locusts, we dissected the midgut in *Sg*-Ringer solution under a binocular microscope. Midgut fragments of equal length were dissected. The entire midgut juice content of these fragments was collected. The midgut content of three or more locusts was dissolved in 100 µl *Sg*-Ringer solution (1 L: 8.766 g NaCl; 0.188 g $CaCl_2$; 0.746 g KCl; 0.407 g $MgCl_2$; 0.336 g $NaHCO_3$; 30.807 g sucrose; 1.892 g trehalose; pH 7.2). After mixing the midgut juice solution, the samples were centrifuged for 3 minutes at 3,000 x rpm and the resulting supernatants was stored at -20°C until further usage.

5.2.4. Quantification of total protein amount in the midgut juice

The total protein concentration in the midgut juice samples was determined according to the BiCinchoninic Acid (BCA) assay. For this purpose, two reagents named A and B were mixed in a 1/49 ratio. Reagent A was prepared by adding sodium bicinchoninate (0.1 g), $Na_2CO_3.H_2O$ (2 g), sodium tartrate (dihydrate) (0.16 g), NaOH (0.4 g) and $NaHCO_3$ (0.95 g) in 100 mL of distilled H_2O and adjusting the pH

to 11.25. Reagent B consisted of 0.4% $CuSO_4.5H_2O$. Bovine serum albumin (BSA) standard solutions were used as reference and the measurements were performed by means of a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc.). Afterwards, *Sg*-Ringer solution was used to dilute the samples to the same concentration.

5.2.5. *In vitro* dsRNA persistency assay

To 10 µl midgut juice or hemolymph sample, we added 150 ng of ds*tubu* or *dsgapdh*. *Sg*-Ringer was used to dilute the samples prior to adding dsRNA. As a control, 150 ng of dsRNA was added to 10 µl *Sg*-Ringer solution. After the indicated incubation time, 2 µl 6x loading dye (Fermentas) was applied. Thereafter, the samples were analysed by means of 1% agarose electrophoresis. The dsRNA was visualized with a ProXima 2500 imager (Isogen Life Science) under UV-light.

In some cases, prior to adding dsRNA, cations in the sample were sequestered by adding 0.05 M ethylenediaminetetraacetic acid (EDTA, Sigma Aldrich co.) for 2 minutes. In addition, nucleoprotein complexes in the samples were dissociated by means of phenol-chloroform extraction. Therefore, we added 30 µl Qiazol (Qiagen), mixed the samples and waited for 5 min. Then, we added 30 µl chloroform, mixed the samples and after 3 minutes incubation at room temperature, they were centrifuged at 13,000 rpm for 15 min at 4°C. The upper phase was collected and the dsRNA was purified by means of ethanol precipitation. Therefore, we added 300 µl 100% ethanol and 10 µl 3 M sodium acetate. These samples were stored at -20°C for 2 hours and subsequently centrifuged for 30 minutes at 4°C. The pellet was washed with 70% ethanol and centrifuged for 15 min. The pellet was then diluted in 10 µl MilliQ water.

5.2.6. Synthesis of dsRNA

Double stranded RNAs for *tubu* (545 bp), *glyceraldehyde 3-phosphate dehydrogenase* (*gapdh*) (447 bp), *green fluorescent protein* (*gfp*) (589 bp), *dsRNase1* (576 bp), *dsRNase2* (346 bp), *dsRNase3* (496 bp), *dsRNase4* (646 bp), *apo1/2* (631 bp) and *apo3* (277 bp) were synthesized using the MEGAscript RNAi kit (Ambion) as described in § 2.4. The primer sequences are also displayed in § 2.4.

5.2.7. Production of Cy3-dsRNA

We produced Cy3-labelled dsRNA by using the Silencer®siRNA labelling kit (Ambion), according to the manufacturer's specifications. The labelled dsRNA molecules were separated from the unbound Cy3-dyes via ethanol precipitation. The Cy3-labelling was confirmed by assessing the fluorescence with an Ethan DIGE imager (GE healthcare), following 1% agarose gel electrophoresis (without adding GelRed (Fermentas)). The dsRNA-concentration and labelling efficiency was determined with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc.).

5.2.8. RNA extraction and cDNA synthesis

The RNA of individual tissues was extracted with the Lipid tissue extraction kit (Qiagen), in accordance to the methods described in § 2.7. The RNA-samples were stored at -80°C. Next, equal quantities of RNA were used as template to produce cDNA. The cDNA synthesis was performed using the First strand cDNA Synthesis Kit (TaKaRa) as described in § 2.7.

5.2.9. Quantitative real time PCR

The knockdown was assessed by means of qPCR in accordance to the described procedures and by using the primer sequences displayed in § 2.8. All data were analysed by non-parametric statistics in GraphPad prism 5 (GraphPad).

5.2.10. Purification and analysis of the gel mobility shift band

The gel mobility shift band was cut out and stored in MagnaLyser Green beads containing tubes (Roche). Next, 1 ml MilliQ water was added to the samples and the agarose gel was fractioned with a MagnaLyser (Roche). The agarose matrix was subsequently separated by means of centrifugation. Next, the remaining agarose in the supernatants was removed via a second centrifugation step. Thereafter, the sample was concentrated by means of RotaVap lyophilisation (FTS Systems, inc.).

The samples were subsequently analysed by means of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), prepared with NuPAGE® Bis-Tris Mini Gels (Life technologies Co.). The staining was performed with SimplyBlue™ Safe

stain (Life technologies Co.) according to the manufacturers' specifications. The used ladder was SeeBlue® Plus2 Pre-Stained Standard (Life technologies Co.).

5.2.11. Determining the amino acid sequences by Edman degradation

Following the separation of the protein bands by means of SDS-PAGE (NuPAGE® Bis-Tris Mini Gels, Life technologies Co.), the proteins were electrotransferred to a polyvinylidene fluoride (PVDF) membrane with the Cell II™ Blot module (Life Technologies Co.). After electrotransfer, the PVDF membranes were stained with amino black staining solution (amido Black (0.5% w/v), isopropanol (25% v/v) and acetic acid (10% v/v)) for 2 min. Destaining was done by several soakings in deionized water. In the lab of Prof. Paul Proost (Rega Institute, KU Leuven), the corresponding amino acid sequences were determined by automated Edman degradation, using a 491 Procise cLC protein sequencer (Applied Biosystems).

5.2.12. Purification of lipophorins

Lipophorins were purified from the hemolymph of desert locusts by ultracentrifugation in a KBr-density gradient. Therefore, in a Polyallomer Centrifugation Tube (Beckman), we diluted the hemolymph sample to a volume of 5 ml with a solution containing 150 mM NaCl and 5 mM EDTA in 50 mM phosphate buffer and with a PH-value of 6.8. On top, we slowly added 5.5 ml 0.9% NaCl, creating in a two-layer solution that was subsequently ultracentrifuged for 10 hours at 50 000 rpm (4°C) using the Optima™ LE-80K untracentrifuge (Beckman). The resulting yellow band was collected with a syringe. Next, the K^+ and Br^- ions were removed from the solution through 24 hours dialysis (using a dialysis bag) in *Sg*-Ringer solution (at 4°C) and the identity of the purified lipophorins was evaluated with SDS-PAGE (NuPAGE® Bis-Tris Mini Gels, Life technologies Co.). The purified lipophorins were stored at -80°C.

5.3. Results

5.3.1. Incubation of dsRNA in midgut juice and serum

The persistence of dsRNA in the hemocoel and gut lumen was assessed by incubating 150 ng of dsRNA for 5 minutes in 10 µl of serum, midgut juice or *Sg*-Ringer solution. Next, the dsRNA was visualized by means of 1% agarose gel electrophoresis. We added two additional controls, namely 10 µl of serum or midgut juice, without adding dsRNA. Hereby, the natural fluorescence of serum and midgut juice was taken into account, *i.e.* in the presence of GelRed (Fermentas) and UV-light. Whereas only limited natural fluorescence was observed in the serum, an intense band was detected in the midgut juice sample (Fig. 1A). Moreover, a similar band was observed in all further experiments with midgut juice. In contrast to the incubation of dsRNA in *Sg*-Ringer solution, incubation in serum or midgut juice resulted in the absence of a dsRNA-band with the expected length of approximately 500 bp. As is the case for many nucleases, sequestering cationic cofactors with 0.05 M EDTA could inhibit the degradation activity in the serum and midgut juice. In this respect, a dsRNA-band of the expected length was observed for the midgut juice sample that was supplemented with EDTA. Yet, following the incubation of dsRNA in serum that was supplemented with EDTA, an intense band with reduced electrophoretic mobility was reported, a phenomenon known as a 'gel mobility shift' (GMS). To assess whether the formation of the GMS was due to dsRNA-binding of a (unidentified) protein, we repeated the previous experiment, but prior to assessing the dsRNA by means of electrophoresis, we first dissociated the nucleoprotein complexes in the solution by means of phenol-chloroform extraction and subsequently purifying the unbound nucleic acids with ethanol precipitation (Fig. 1B). This resulted in the disappearance of the GMS and in a thin band of intact dsRNA for the serum sample. In addition, we confirmed the fact that sequestering cations with EDTA inhibited the degradation of dsRNA in serum and midgut juice samples, since adding EDTA increased the intensity of the bands.

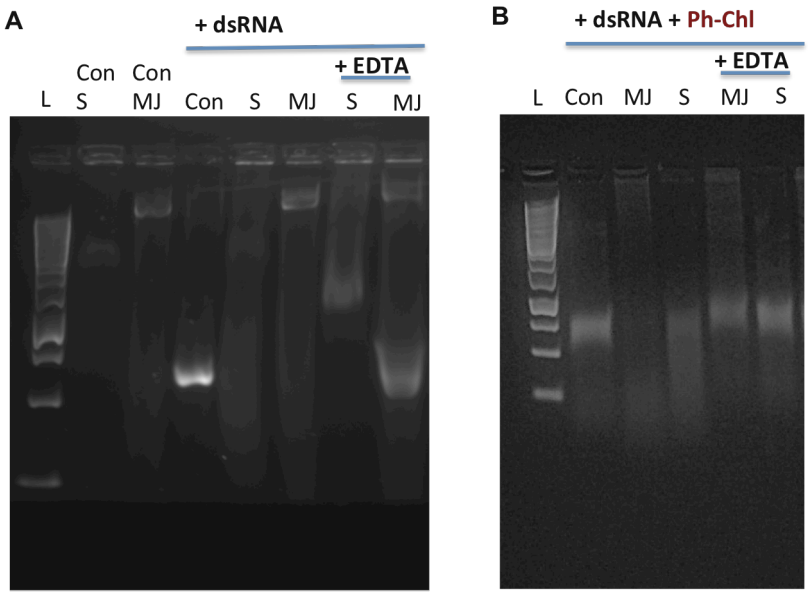


Fig. 1. Following the incubation of 150 ng of dsRNA in 10 µl serum, midgut juice or *Sg*-Ringer solution for 5 minutes, the dsRNA was analysed by means of 1% agarose electrophoresis. (A) By administering pure serum (Con S) or dissolved midgut juice (Con MJ), we assessed the autofluorescence (in the presence GelRed (Fermentas) and UV-light) in these samples, which should be taken into account for correct data analyses. The dsRNA was incubated in *Sg*-Ringer solution (Con), serum (S) or midgut juice (MJ). In addition, by adding 0.05 M EDTA (+ EDTA), free cations were depleted from the serum and midgut juice. Two minutes later, the 150 ng of dsRNA was added to the samples and incubated for 5 minutes. (B) Prior to electrophoresis, the nucleoprotein complexes were dissociated by means of phenol chloroform extraction (Ph-chl).

Next, 150 ng dsRNA was incubated in a serial dilution of serum (diluted with *Sg*-Ringer solution) (Fig. 2A). Interestingly, the intensity of the GMS-band markedly increased for diluted serum samples and the electrophoretic mobility changed for different serum dilutions. To check whether the long dsRNA was converted into active siRNAs, we also added a 20 bp ladder (Fermentas). Yet, there was no clear band with a size of approximately 21 bp observable. Dissociation of the nucleoprotein complexes in these samples by means of phenol-chloroform extraction resulted in a marked increase in intensity of the dsRNA-band with the dilution of the samples (fig 3B). In contrast, after incubation of dsRNA in a serial dilution of midgut juice, there was no GMS-band observable.

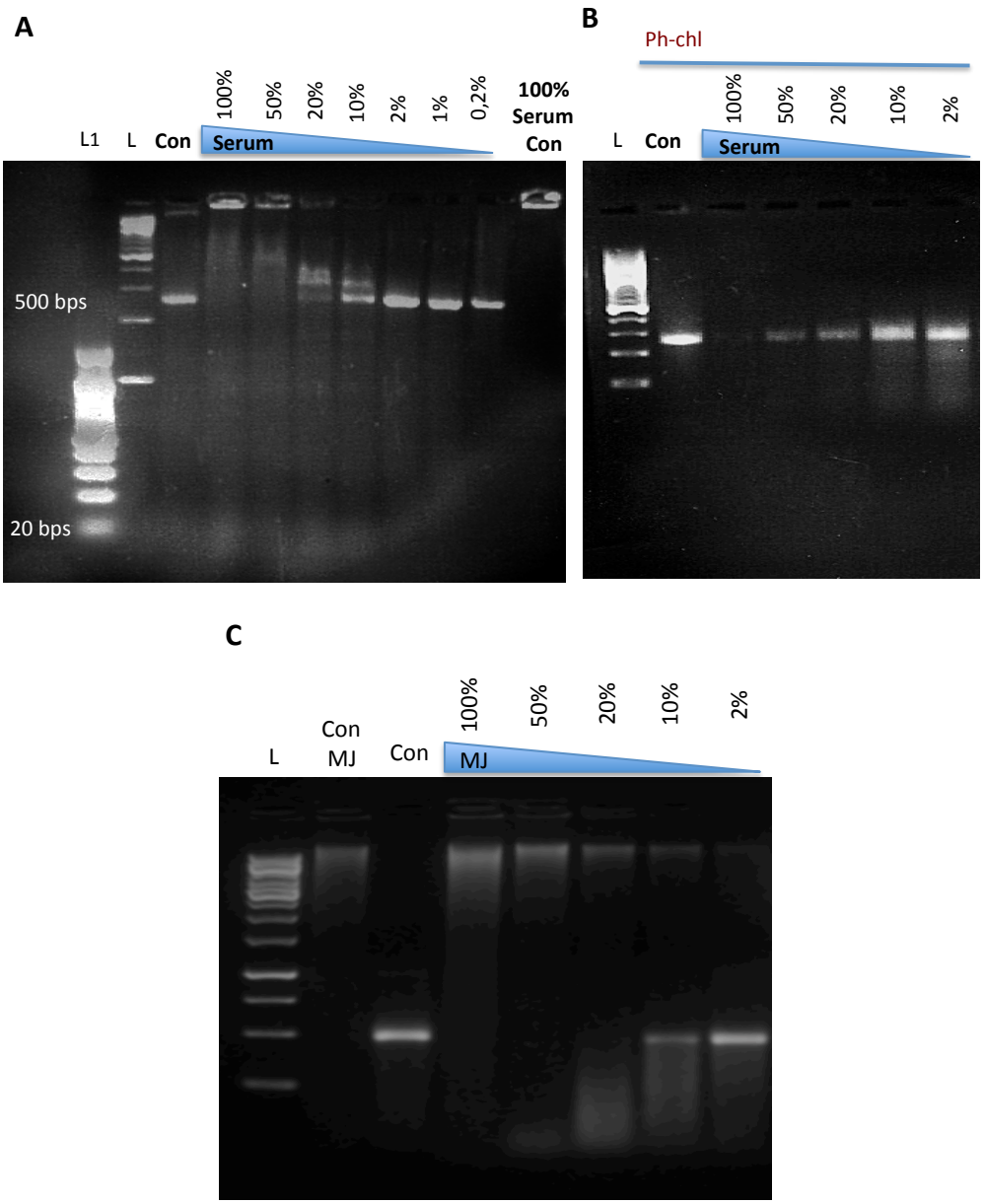


Fig. 2. Incubation of 150 ng of dsRNA in different serum or midgut juice concentrations for 5 minutes. (A) The samples were directly analysed by means of 1% agarose gel electrophoresis. A 20 bp ladder (L1) was added to assess the formation of siRNA fragments with a size of approximately 21 bp and a 200 bp ladder (L) was applied to assess the integrity of intact dsRNA (500 bp). The autofluorescence in the serum sample (100% serum con) was taken into account, as well as a control reaction (con) where the dsRNA was incubated in *Sg*-Ringer solution. (B) The nucleoprotein complexes were first dissociated by means of phenol chloroform extraction (ph-chl) before analysing the dsRNA-integrity via electrophoresis. (C) The integrity of the dsRNA following its incubation in different midgut juice (MJ) concentrations. Pure MJ was also assessed (Con MJ), as well as a control reaction, where the dsRNA was incubated in *Sg*-Ringer solution (Con).

5.3.2. Identification of four dsRNases in the *S. gregaria* transcriptome database

In order to identify the nucleases that may be responsible for the degradation of dsRNA in the midgut juice and serum of *S. gregaria*, a candidate gene approach was employed based on the *B. mori alkaline nuclease* that is secreted into the midgut lumen, where it can digest dsRNA (Arimatsu *et al.*, 2007). Four different transcript sequences with high sequence similarity to the *alkaline nuclease* of *B. mori* were found in the transcriptome database of *S. gregaria* and were termed *dsRNase1*, *-2*, *-3* and *-4*. The deduced amino acid sequences were predicted to contain a single DNA/RNA non-specific nuclease (NN-)domain (Pfam). For two *dsRNases* (*dsRNase1* and *dsRNase4*), the complete ORF sequence was available and the translated transcripts were predicted to possess a signal peptide sequence (determined by using the SignalP 4.1 software) (Fig. 3).

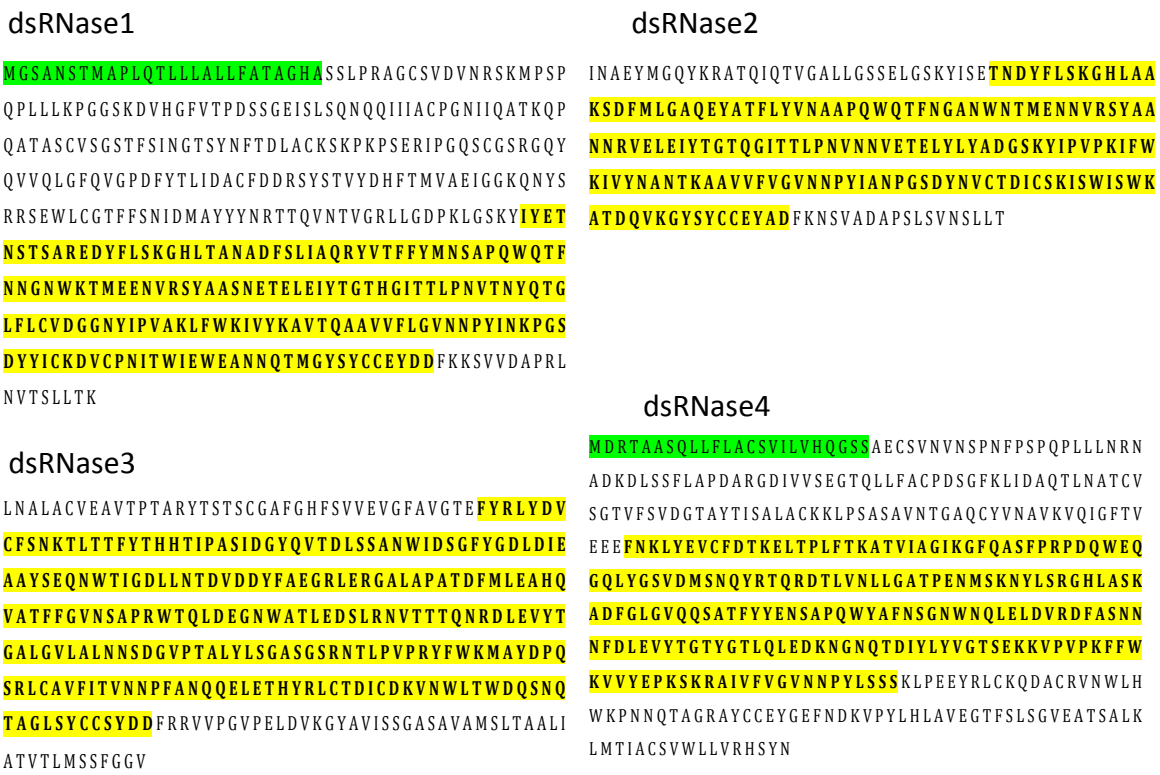


Fig. 3. The deduced amino acid sequence of the four *dsRNase* transcript sequences present in the transcriptome database of the desert locust. The predicted NN-domain (Pfam) is indicated in yellow and the signal peptide (SignalP 4.1) in green.

5.3.3. Functional characterization of the dsRNases

By means of qPCR, we determined the transcript tissue distribution of the *dsRNases* in seven-day old adult desert locusts. Our experimental data demonstrate that they are mainly expressed by the midgut (Fig. 4).

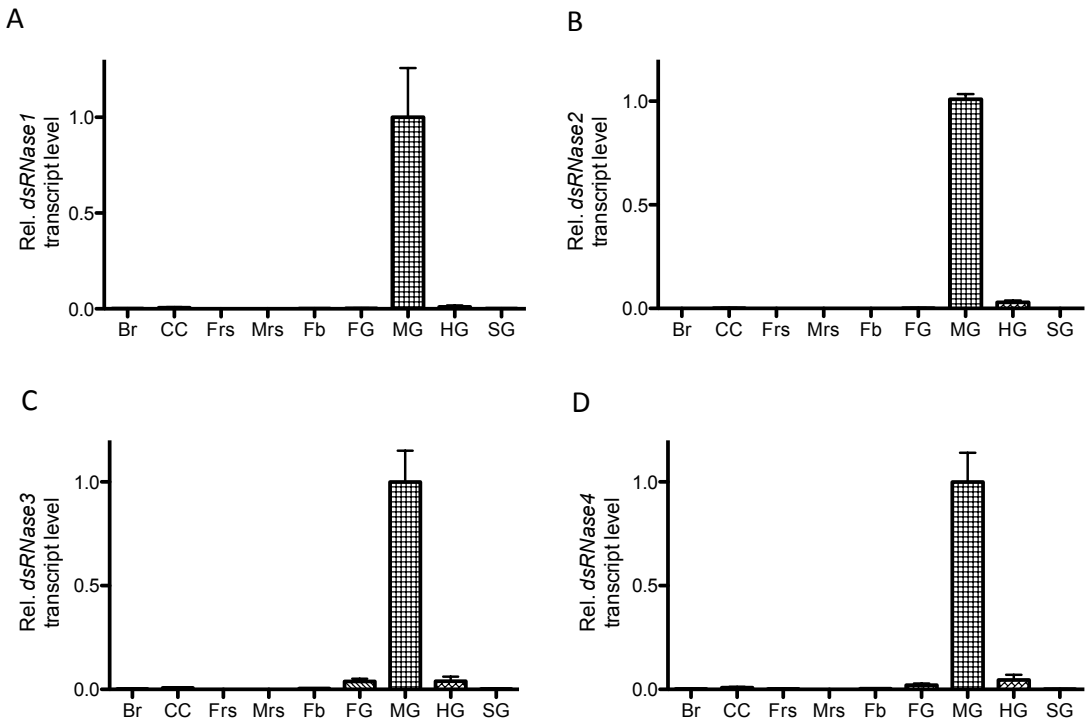
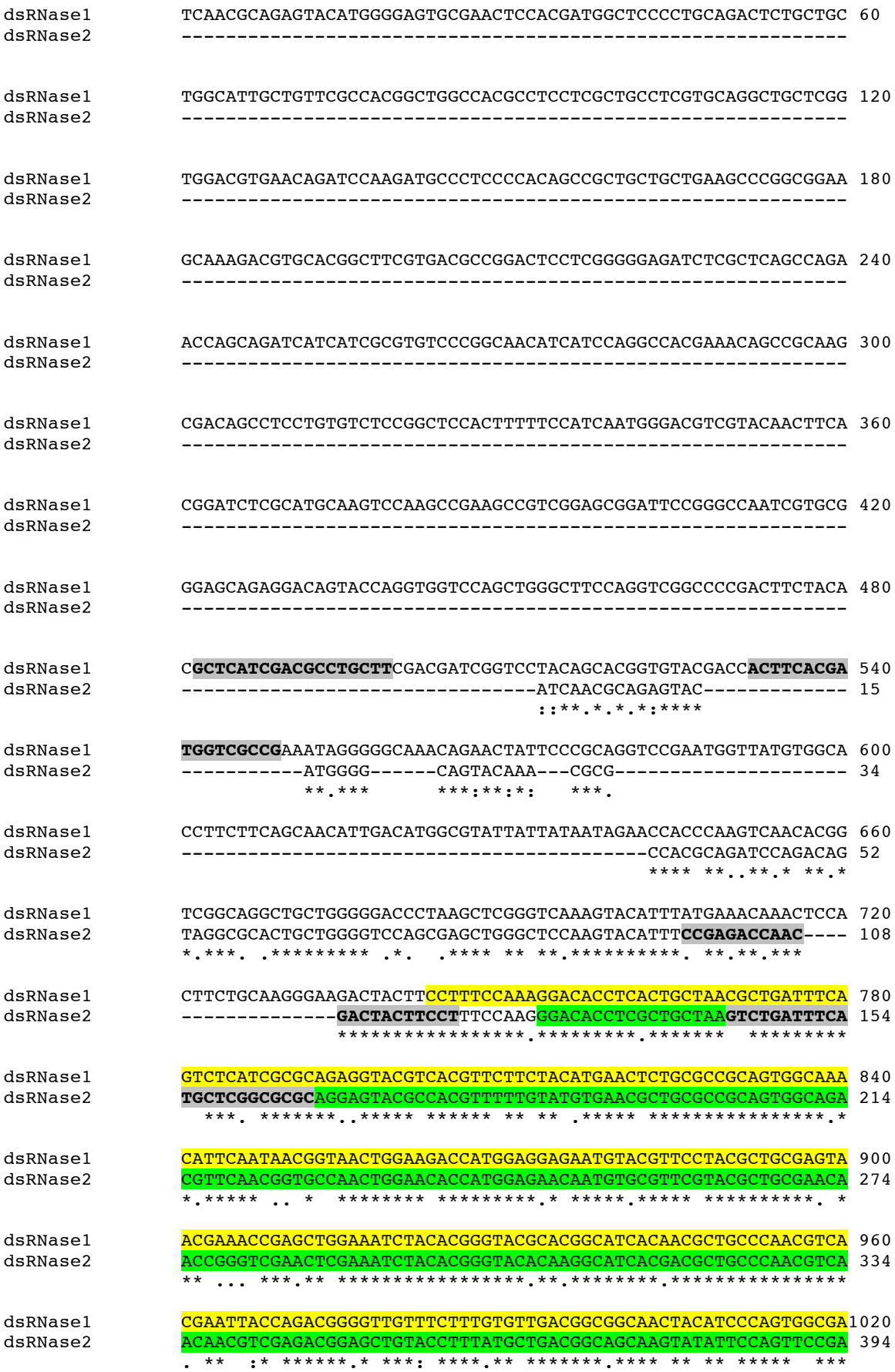


Fig. 4. The transcript profile of the *dsRNases* in seven-day old adult desert locusts: for (A) *dsRNase1*, (B) *dsRNase2*, (C) *dsRNase3* and (D) *dsRNase4*. Each bar represents the mean ± SEM of two independent pools of adult males (40 and 10 animals/pool) and two independent pools of adult females (40 and 10 animals/pool), (Br= brain, CC= corpora cardiata, Frs= female reproductive system, Mrs= male reproductive system, Fb= fat body, FG= foregut, MG= midgut, HG= hindgut and SG= salivary glands).

Next, we tested whether silencing the *dsRNases* by means of RNAi could reduce the dsRNA-degrading activity in the midgut juice or serum. However, due to high transcript sequence similarity (Fig. 5), silencing individual *dsRNases* was challenging.

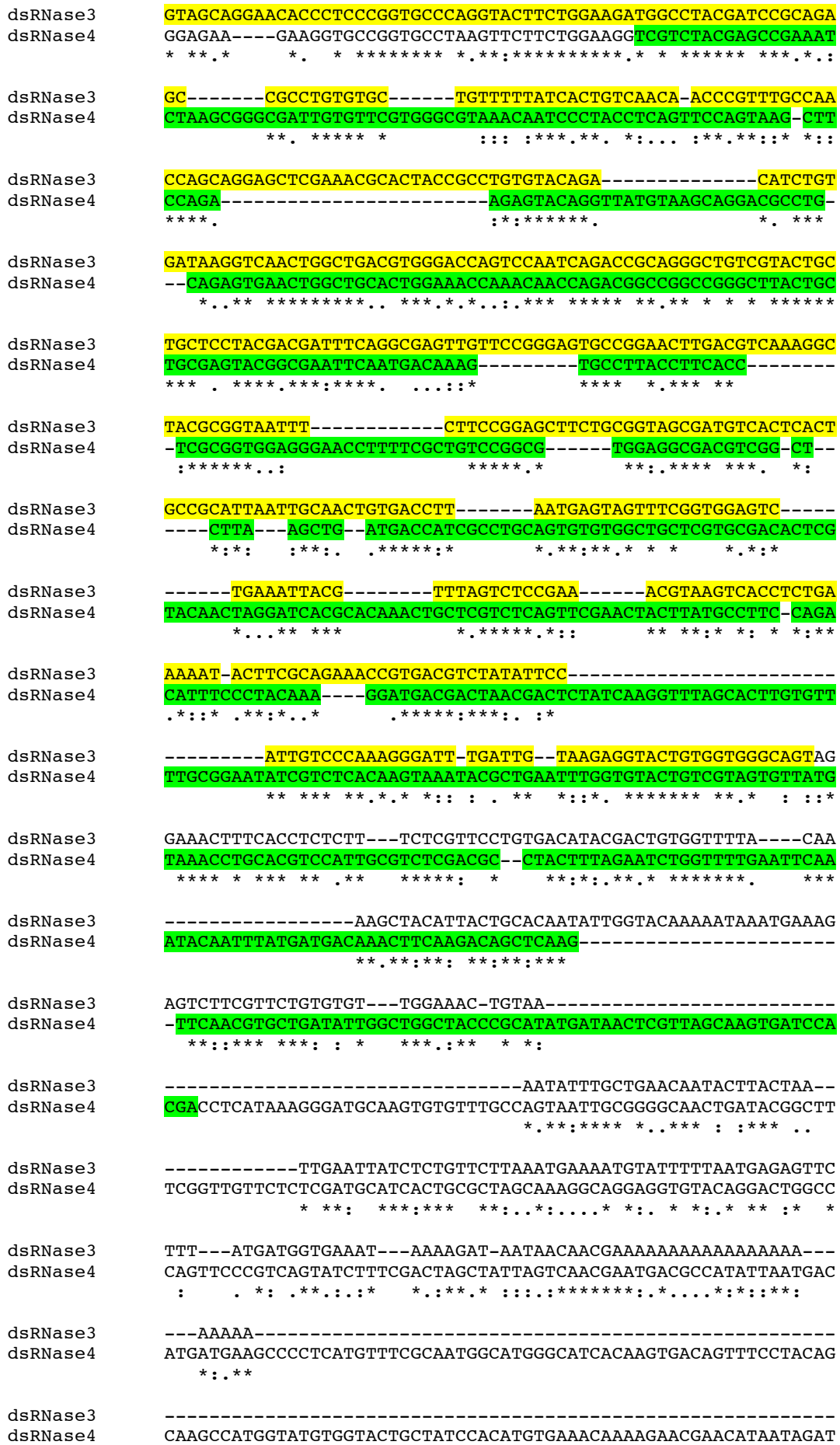
A


dsRNase1 TCAACGCAGAGTACATGGGGAGTGCGAACTCCACGATGGCTCCCCTGCAGACTCTGCTGC 60
dsRNase2 --
dsRNase1 TGGCATTGCTGTTCGCCACGGCTGGCCACGCCTCCTCGCTGCCTCGTGCAGGCTGCTCGG 120
dsRNase2 --
dsRNase1 TGGACGTGAACAGATCCAAGATGCCCTCCCCACAGCCGCTGCTGCTGAAGCCCGGCGGAA 180
dsRNase2 --
dsRNase1 GCAAAGACGTGCACGGCTTCGTGACGCCGGACTCCTCGGGGGAGATCTCGCTCAGCCAGA 240
dsRNase2 --
dsRNase1 ACCAGCAGATCATCATCGCGTGTCCCGGCAACATCATCCAGGCCACGAAACAGCCGCAAG 300
dsRNase2 --
dsRNase1 CGACAGCCTCCTGTGTCTCCGGCTCCACTTTTTCCATCAATGGGACGTCGTACAACTTCA 360
dsRNase2 --
dsRNase1 CGGATCTCGCATGCAAGTCCAAGCCGAAGCCGTCGGAGCGGATTCCGGGCCAATCGTGCG 420
dsRNase2 --
dsRNase1 GGAGCAGAGGACAGTACCAGGTGGTCCAGCTGGGCTTCCAGGTCGGCCCCGACTTCTACA 480
dsRNase2 --
dsRNase1 CGCTCATCGACGCCTGCTTCGACGATCGGTCCTACAGCACGGTGTACGACCACTTCACGA 540
dsRNase2 --------------------------------ATCAACGCAGAGTAC------------- 15
::**.*.*.*:****
dsRNase1 TGGTCGCCGAAATAGGGGGCAAACAGAACTATTCCCGCAGGTCCGAATGGTTATGTGGCA 600
dsRNase2 -----------ATGGGG------CAGTACAAA---CGCG---------------------- 34
.* ***:**:*: ***.
dsRNase1 CCTTCTTCAGCAACATTGACATGGCGTATTATTATAATAGAACCACCCAAGTCAACACGG 660
dsRNase2 ---CCACGCAGATCCAGACAG 52
**** **..**.* **.*
dsRNase1 TCGGCAGGCTGCTGGGGGACCCTAAGCTCGGGTCAAAGTACATTTATGAAACAAACTCCA 720
dsRNase2 TAGGCGCACTGCTGGGGTCCAGCGAGCTGGGCTCCAAGTACATTTCCGAGACCAAC---- 108
*.***. .********* .*. .**** ** **.**********. **.**.***
dsRNase1 CTTCTGCAAGGGAAGACTACTTCCTTTCCAAAGGACACCTCACTGCTAACGCTGATTTCA 780
dsRNase2 --------------GACTACTTCCTTTCCAAGGGACACCTCGCTGCTAAGTCTGATTTCA 154
**********.*********.******* *********
dsRNase1 GTCTCATCGCGCAGAGGTACGTCACGTTCTTCTACATGAACTCTGCGCCGCAGTGGCAAA 840
dsRNase2 TGCTCGGCGCGCAGGAGTACGCCACGTTTTTGTATGTGAACGCTGCGCCGCAGTGGCAGA 214
. ****..***** ****** ** ** .***** ***************.*
dsRNase1 CATTCAATAACGGTAACTGGAAGACCATGGAGGAGAATGTACGTTCCTACGCTGCGAGTA 900
dsRNase2 CGTTCAACGGTGCCAACTGGAACACCATGGAGAACAATGTGCGTTCGTACGCTGCGAACA 274
*.***** .. * ******** *********.* *****.***** **********. *
dsRNase1 ACGAAACCGAGCTGGAAATCTACACGGGTACGCACGGCATCACAACGCTGCCCAACGTCA 960
dsRNase2 ACCGGGTCGAACTCGAAATCTACACGGGTACACAAGGCATCACGACGCTGCCCAACGTCA 334
** ... ***.** **********.**.********.***************
dsRNase1 CGAATTACCAGACGGGGTTGTTTCTTTGTGTTGACGGCGGCAACTACATCCCAGTGGCGA1020
dsRNase2 ACAACGTCGAGACGGAGCTGTACCTTTATGCTGACGGCAGCAAGTATATTCCAGTTCCGA 394
. ** :* ******.* ***: ****.** *******.**** ** ** ***** ***

```
dsRNase1        AGCTCTTCTGGAAGATTGTGTATAAGGCAGTTACTCAGGCAGCTGTAGTGTTCCTGGGTG1080
dsRNase2        AGATCTTCTGGAAAATCGTGTATAACGCTAACACTAAGGCAGCCGTTGTGTTCGTGGGCG 454
                **.**********.** ******** **:.: ***.******* **:****** **** *

dsRNase1        TAAACAACCCGTACATCAATAAACCTGGCTCTGACTACTACATCTGCAAAGACGTATGCC1140
dsRNase2        TCAACAATCCGTACATCGCGAACCCCGGATCTGACTACAATGTGTGCACAGACATATGTA 514
                *.***** *********.. **.** **.*********:* .* ****.****.**** .

dsRNase1        CCAACATCACCTGGATAGAATGGGAGGCCAATAACCAAACGATGGGGTACTCGTACTGCT1200
dsRNase2        GCAAGATCAGTTGGATATCCTGGAAGGCCACGGATCAGGTGAAGGGCTACTCGTACTGTT 574
                 *** ****  ****** ..***.******. .* **.. **:*** ********** *

dsRNase1        GTGAATATGATGACTTCAAGAAATCTGTTGTCGACGCACCCAGACTCAACGTTACCTCTC1260
dsRNase2        GCGAGTATGCAGACTTCAAGAACTCCGTGGCCGACGCACCCAGCCTCAGCGTTAACTCTT 634
                * **.****.:**********.** ** * ***********.****.*****.****

dsRNase1        TCCTTACGAAATAGTCTCAACTGGATGGCAGTTCGAGTTATCTTCAAAAACCCGCAGGAA1320
dsRNase2        TGCTTACCTAATC-TCCCAGATG---GGAAATACTT-TTGTCTGAAGATATCAGGTGGAA 689
                * ***** :***. ** **..**   **.*.*:* : **.*** .*.*:* *.* :****

dsRNase1        CTCACACCAAACGAAAACCGCATTCAAACCGTCTACGGGATCCAACAAG-------GAAT1373
dsRNase2        GCCACACCAGACGAACACATTACTCAGCACTTATAGTGAATCCAATAAGAAATCTAGAAC 749
                  *******.*****.**.  * ***...* *.**  *.****** ***       ***

dsRNase1        GTAAATATATCTGCAACTCTTTATTTCGGTTAGTTCACAGTCTTTGAACTTTGGCGATAT1433
dsRNase2        GTTCATACATTTGTAACTCTGAATACCAGATACACCATAGTCCAGTAAATT----GAAAT 805
                **:.*** ** ** ****** :**: *.*:** : ** **** :  **.**    **:**

dsRNase1        CCATATTTTAATTGTATCTTCTTTGTGCCTTCTTTGAAGACATAAGCCTAAAGTTAAGAG1493
dsRNase2        CCATATTTGAACTGTATCGTTTTTGTTCCTTCTTTGTACATCTATGCT-----TTAAGTG 860
                ******** ** ****** * ***** *********:* * .**:**      *****:*

dsRNase1        GTAATTGTGGCTTTTGATTTACATATTCCGCCTCTCTCTCTGAAATTTATTTCTACCAGT1553
dsRNase2        ATTATACTGGCTTTTGATTGA---------------------------------------- 881
                .*:**: ************ *

dsRNase1        TTAAGTAAAGCTGTCCAGTACGAATAATTTTGTTCCATTTATGACACGTTTTATTTTTAG1613
dsRNase2        ------------------------------------------------------------

dsRNase1        ATGACAGCATGGGAATGCTAGACTTTATGTATATATGATATATGAAACACAGCAAATTTC1673
dsRNase2        ------------------------------------------------------------

dsRNase1        ACAGTTTTATCCGATTTTACGTCTTAGTTGCATAATTACAGGACAAGGTTAGAGTGGAAA1733
dsRNase2        ------------------------------------------------------------

dsRNase1        AAACAAGATGAGTGTAAATAACAGCAAAATCTATATAAAGTTATTCTTAACCTAAACGTT1793
dsRNase2        ------------------------------------------------------------

dsRNase1        ATGTGTATGTATGCATTTCAGAAAAATAAAAGAAAATATCCATTGATTATGCCACAAAAA1853
dsRNase2        ------------------------------------------------------------

dsRNase1        AAAAAAAAAAAAAAAAAAA 1872
dsRNase2        -------------------
```

B

```
dsRNase3      ------------------------------------------------------------
dsRNase4      GTATCAACGCAGAGTACATGGGGATTCTCCGAGAGACGCTTGTGATTGCACACCGAACAT

dsRNase3      ------------------------------------------------------------
dsRNase4      CATGGATCGAACGGCAGCTTCCCAACTCCTCTTCCTGGCGTGTTCCGTCATTCTGGTCCA

dsRNase3      ------------------------------------------------------------
dsRNase4      TCAAGGCAGCTCTGCAGAGTGCTCGGTGAACGTAAACTCTCCGAATTTTCCGTCGCCTCA

dsRNase3      ------------------------------------------------------------
dsRNase4      ACCGCTGCTGCTCAACCGAAACGCAGACAAGGACCTGAGCAGCTTCCTGGCGCCGGACGC

dsRNase3      ------------------------------------------------------------
dsRNase4      ACGCGGCGACATCGTGGTCAGCGAGGGCACGCAGCTGCTGTTCGCCTGTCCTGACTCCGG

dsRNase3      --------CTCTCAACGCGCTGGCG--------------------------------TGTG
dsRNase4      GTTCAAGCTGATCGACGCGCAGACTCTGAATGCCACCTGCGTCTCTGGCACCGTCTTCAG
                        .**.******:*.*                                 * :*

dsRNase3      TCGAGGCTGTGACGCC--------CACGGCGCGCTACACCAGCACCTCGTGCGGCGCCTT
dsRNase4      CGTAGACGGAACCGCCTACACGATCTCAGCACTCGCCTGCAAGAAGCTGCCCAGCGCCAG
                 **.* *:..****        *:*.**.* * .*: **. *.   *  *.*****:

dsRNase3      CGGACACTTCTCCGTCG---------------T------------CGAGGTGGGTTTCGC
dsRNase4      CGCTGTCAACACCGGCGCACAGTGCTATGTCAATGCAGTAAAGGTACAGATCGGCTTCAC
              ** : :*::*:*** **               :            . **.* ** ***.*

dsRNase3      TGTGGGCACCGAGTTCTACCGCCTGTACGACGTTTGCTTCTCCAACAAGACCTTGACCAC
dsRNase4      GGTGGAGGAGGAGTTCAACAAACTGTACGAAGTGTGCTTCGACACGAAGGAGCTGACACC
               ****. .. ******:**...********.** ****** .**. ***..  ****..*

dsRNase3      TTTCTACACTCATCACACCATCCCCGCCTCCATCGACGGGTATCAGGTGACTGATCTCTC
dsRNase4      GTTGTTTACAAAAGCCACCGTCATCGCCGGCATCAAGGGCTTCCAGGCCTCCTTCCCCA-
               ** *: **:.*: .****.**. ****  ****.* ** *: ****  :*  : * *:

dsRNase3      GTCGGCCAACTGGATCGACAGCGGCTTCTACGGGGATCTGG-ACATCGAAG-----CTGC
dsRNase4      -------GACCCGATCA------------AT-GGGAGCAGGGCCAACTGTACGGCTCAGT
                     .**  ****.            *  **** *:** .**:* .:.     *:*

dsRNase3      GTA---CTCGGAACAGAACTGGACCATTGGCG--ACCTACTTAACACAGACGTTGACGAC
dsRNase4      GGACATGTCAAACCAGTACAGGACTCAGCGGGACACCCTCGTGAAT--------------
              * *    **..*.***:**:**** .:  * *  *** :* *.*.:

dsRNase3      TACTTCGCCGAGGGTCGCCTG----------------------GAGAGAGGCGCACTCG
dsRNase4      --CTACTAGGTGCGACGCCCGAGAACATGAGCAAGAACTACCTCAGCAGAGGACACCTGG
                **:* . *:* *:**** *                       .. *****. ..** *

dsRNase3      CCCCAGCGACAGACTTCATGCTGGAAGCGCATCAAGTGGCCACCTTCTTCGGCGTCAACT
dsRNase4      CCTCCAAGGCGGACTTCGGGCTGGGCGTGCAGCAGTCGGCTACCTTCTACTACGAAAACT
              ** *...*.*.******. *****..* *** **.  *** *******:* .**:.****

dsRNase3      CGGCTCCGCGCTGGAC-GCAGCTGGACGAGGGCAACTGGGCGACGCTCGAGGACAGCCTG
dsRNase4      CGGCCCCACAGTGGTACGCC-TTCAACTCCGGCAACTGGAACCAGCTGGAGTTGGACGTG
              **** **.*. ***:. **.  * .** . *********.. ..*** *** : ..* **

dsRNase3      CGCAACGTGAC-GACGACACAAAACAGGGACCTGGAGGTGTACACCGGTGCCTTAGGCGT
dsRNase4      CGCGACTTCGCCAGCAA-CAACAACTTCGACCTTGAGGTGTACACGGGCACTTACGGTAC
              ***.** * .* ..*.* ..*.***:  ***** ********** ** .* *:.** .

dsRNase3      TCTTGCGCTCAACAACAGCGACGGCGTGCCTACGGCGCTCTACCTG-TCGGGGGCTAGCG
dsRNase4      ACTGCAACTCGAGGACAAGAACGGCAACCAGACGGACATCTACTTATACGTAGGCACGTC
              :**  ..***.* .***. .*****.: *. ****. .***** *. :** .***:.*
```


dsRNase3 GTAGCAGGAACACCCTCCCGGTGCCCAGGTACTTCTGGAAGATGGCCTACGATCCGCAGA
dsRNase4 GGAGAA----GAAGGTGCCGGTGCCTAAGTTCTTCTGGAAGGTCGTCTACGAGCCGAAAT
dsRNase3 GC-------CGCCTGTGTGC------TGTTTTTATCACTGTCAACA-ACCCGTTTGCCAA
dsRNase4 CTAAGCGGGCGATTGTGTTCGTGGGCGTAAACAATCCCTACCTCAGTTCCAGTAAG-CTT
dsRNase3 CCAGCAGGAGCTCGAAACGCACTACCGCCTGTGTACAGA--------------CATCTGT
dsRNase4 CCAGA------------------------AGAGTACAGGTTATGTAAGCAGGACGCCTG-
dsRNase3 GATAAGGTCAACTGGCTGACGTGGGACCAGTCCAATCAGACCGCAGGGCTGTCGTACTGC
dsRNase4 --CAGAGTGAACTGGCTGCACTGGAAACCAAACAACCAGACGGCCGGCCGGGCTTACTGC
dsRNase3 TGCTCCTACGACGATTTCAGGCGAGTTGTTCCGGGAGTGCCGGAACTTGACGTCAAAGGC
dsRNase4 TGCGAGTACGGCGAATTCAATGACAAAG---------TGCCTTACCTTCACC--------
dsRNase3 TACGCGGTAATTT------------CTTCCGGAGCTTCTGCGGTAGCGATGTCACTCACT
dsRNase4 -TCGCGGTGGAGGGAACCTTTTCGCTGTCCGGCG------TGGAGGCGACGTCGG-CT--
dsRNase3 GCCGCATTAATTGCAACTGTGACCTT-------AATGAGTAGTTTCGGTGGAGTC-----
dsRNase4 ----CTTA---AGCTG--ATGACCATCGCCTGCAGTGTGTGGCTGCTCGTGCGACACTCG
dsRNase3 ------TGAAATTACG--------TTTAGTCTCCGAA------ACGTAAGTCACCTCTGA
dsRNase4 TACAACTAGGATCACGCACAAACTGCTCGTCTCAGTTCGAACTACTTATGCCTTC-CAGA
dsRNase3 AAAAT-ACTTCGCAGAAACCGTGACGTCTATATTCC------------------------
dsRNase4 CATTTCCCTACAAA----GGATGACGACTAACGACTCTATCAAGGTTTAGCACTTGTGTT
dsRNase3 ---------ATTGTCCCAAAGGGATT-TGATTG--TAAGAGGTACTGTGGTGGGCAGTAG
dsRNase4 TTGCGGAATATCGTCTCACAAGTAAATACGCTGAATTTGGTGTACTGTCGTAGTGTTATG
dsRNase3 GAAACTTTCACCTCTCTT---TCTCGTTCCTGTGACATACGACTGTGGTTTTA----CAA
dsRNase4 TAAACCTGCACGTCCATTGCGTCTCGACGC--CTACTTTAGAATCTGGTTTTGAATTCAA
dsRNase3 -----------------AAGCTACATTACTGCACAATATTGGTACAAAAATAAATGAAAG
dsRNase4 ATACAATTTATGATGACAAACTTCAAGACAGCTCAAG-----------------------
dsRNase3 AGTCTTCGTTCTGTGTGT---TGGAAAC-TGTAA--------------------------
dsRNase4 -TTCAACGTGCTGATATTGGCTGGCTACCCGCATATGATAACTCGTTAGCAAGTGATCCA
dsRNase3 --------------------------------AATATTTGCTGAACAATACTTACTAA--
dsRNase4 CGACCTCATAAAGGGATGCAAGTGTGTTTGCCAGTAATTGCGGGGCAACTGATACGGCTT
dsRNase3 ------------TTGAATTATCTCTGTTCTTAAATGAAAATGTATTTTTAATGAGAGTTC
dsRNase4 TCGGTTGTTCTCTCGATGCATCACTGCGCTAGCAAAGGCAGGAGGTGTACAGGACTGGCC
dsRNase3 TTT---ATGATGGTGAAAT---AAAAGAT-AATAACAACGAAAAAAAAAAAAAAAAA---
dsRNase4 CAGTTCCCGTCAGTATCTTTCGACTAGCTATTAGTCAACGAATGACGCCATATTAATGAC
dsRNase3 ---AAAAA---
dsRNase4 ATGATGAAGCCCCTCATGTTTCGCAATGGCATGGGCATCACAAGTGACAGTTTCCTACAG
dsRNase3 --
dsRNase4 CAAGCCATGGTATGTGGTACTGCTATCCACATGTGAAACAAAAGAACGAACATAATAGAT

```
dsRNase3      ------------------------------------------------------------
dsRNase4      GACTGCACTAATATCAACAAATGTAACAACGTAATGTTGAAATATAGCTTCTCCGTTTCA

dsRNase3      ------------------------------------------------------------
dsRNase4      CACATAAAGAAATTGCTTCATAAAATTGATTGTAGTACTATGTAGTTCACATTTAGGTAT

dsRNase3      ------------------------------------------------------------
dsRNase4      GACTTTCAGAATAAAATGCAGCAAATGAAGCTAACGAACTGAAACGTGATAGACTGAATG

dsRNase3      ------------------------------------------------------------
dsRNase4      TACCTCACTAACCATGGTGTCTAACAAACATTGCTGCTTTTTGGTTTATGTATGTTGCTC

dsRNase3      ------------------------------------------------------------
dsRNase4      TTTAATTTGTGTGTATAACGAATAAAAAGTATTTAATATCACAAATCGATGAAATTGTTA

dsRNase3      ------------------------------------------------------------
dsRNase4      TCAGTTTACCTATACATATTTGAATAAAGATATGAAATAAAATAAAACTAAAAGTTCAAG

dsRNase3      ------------------------
dsRNase4      AAAAAAAAAAAAAAAAAAAAAAA
```

Fig. 5. Comparison of (A) *dsRNase1* and *-2*, and (B) *dsRNase3* and *-4* available transcript sequences present in the *S. gregaria* transcriptome nucleotide database by using the Clustal Omega Sequence Alignment software (EMBL-EBI). The dsRNA fragments directed against *dsRNase1* and *-3* are indicated in yellow, while the dsRNA fragments that target *dsRNase2* and *-4* are designated in green. The qPCR primer sequences are indicated in grey.

For instance, injection of dsRNA for *dsRNase1* also significantly reduced the *dsRNase2* transcript level and injection of *dsRNase2* dsRNA resulted in a significant reduction of the *dsRNase1* transcript level (Fig. 6). Nonetheless, the knockdown was most robust for the dsRNA-specific transcripts. Although it may be expected that the transcript levels of the *dsRNases* were already reduced one day after the injection of dsRNA, we waited six days after the dsRNA-injection, to acquire a knockdown at the resulting protein level.

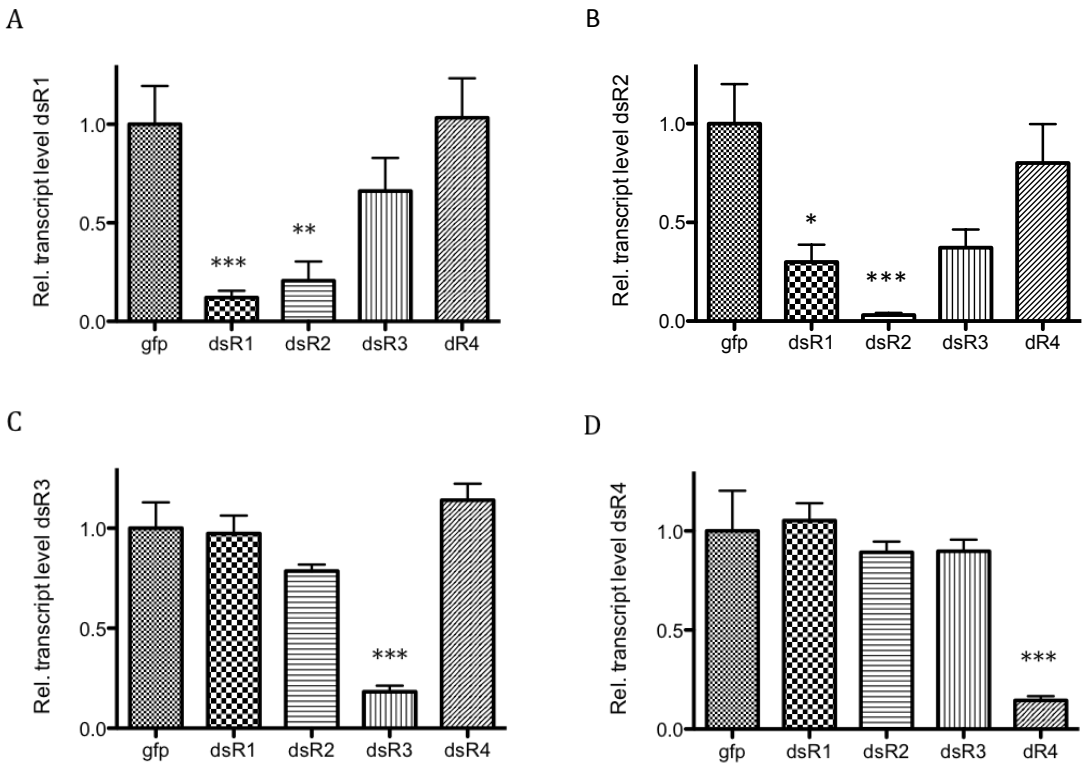
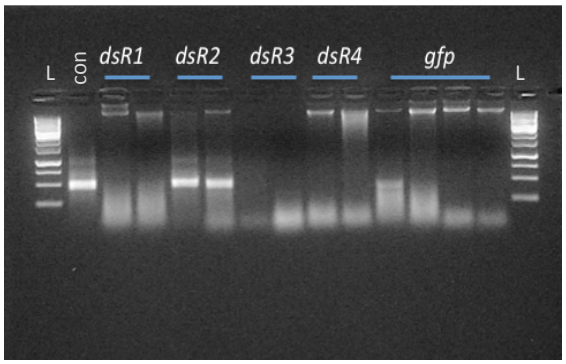


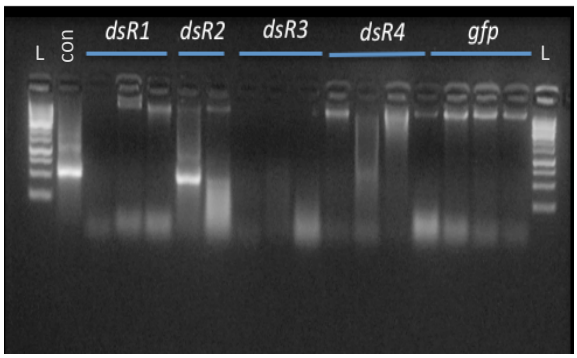
Fig. 6. Injection of four days old adult desert locusts with 150 ng of dsRNA for the different *dsRNases* and the effect on the transcript level in the midgut for (A) *dsRNase1*, (B) *dsRNase2*, (C) *dsRNase3* and (D) *dsRNase4*, six days after the dsRNA-injection (mean ± SEM, n = 4-6; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$).

Injection of *dsRNase2* dsRNA caused a clear reduction in the dsRNase-activity in the midgut juice (Fig. 7A). Moreover, intact dsRNA was still visible after incubation of 150 ng of dsRNA for 15 minutes in midgut juice of locusts that were injected with dsRNA for *dsRNase2* (Fig. 7B). Repeating these experiments also resulted in a small difference in the dsRNase-activity in the midgut juice of locusts that were injected with *dsRNase1* dsRNA. Apparently, the effect was more dramatic for locusts injected with *dsRNase2* dsRNA (Fig. 7C).

A: 3 min incubation:



B: 15 minutes incubation:



C: 3 min incubation:

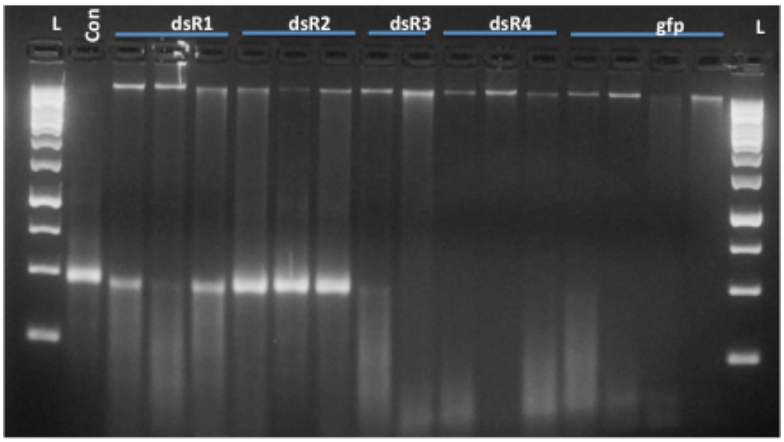


Fig. 7. dsRNase-activity in the midgut juice after silencing *dsRNases*. Four days old adult locusts were injected with dsRNA for *dsRNase1* (dsR1), *dsRNase2* (dsR2), *dsRNase3* (dsR3), *dsRNase4* (dsR4) or *gfp* and six days later the midgut juice was collected, the protein content was determined by means of the BSA method, all samples were diluted to the same protein concentration with *Sg*-Ringer solution and subsequently analysed for dsRNase-activity, *i.e.* (A) for an incubation time of 3 minutes and (B) for an incubation time of 15 minutes. Each lane represents 150 ng of dsRNA that was incubated in the pooled midgut juices of 3 or 4 individual adult desert locusts. (C) In order to validate the reproducibility of these data, new locusts were injected with 150 ng dsRNA (for *dsRNase1* (dsR1), *dsRNase2* (dsR2), *dsRNase3* (dsR3), *dsRNase4* (dsR4) or *gfp*), midgut juice was collected six days later, the protein concentration was determined by means of the BSA method, the samples were diluted with *Sg*-Ringer solution and analysed for dsRNase-activity. Each lane represents 150 ng of dsRNA that was incubated for 3 minutes in the pooled midgut juices of 3 individual adult desert locusts (L = DNA ladder, con= dsRNA incubated in *Sg*-Ringer).

The next logical step is silencing the dsRNase-activity by means of RNAi and subsequently assessing whether orally delivered dsRNA can induce gene silencing. Therefore, the locusts were injected with 150 ng of *dsRNase2* or *gfp* dsRNA and after six days 2 µg *gapdh* or *gfp* dsRNA was orally injected. Yet, three days later, the *gapdh* transcript levels were not down regulated in these locusts (Fig. 8).

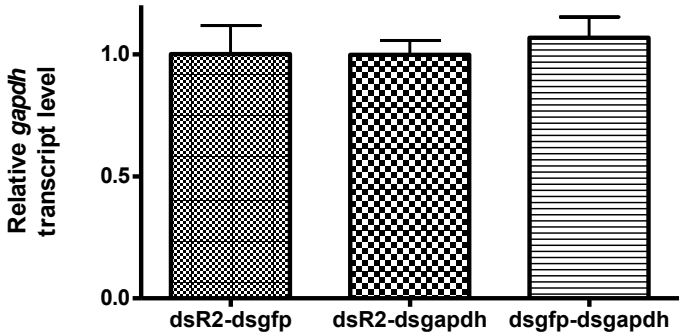


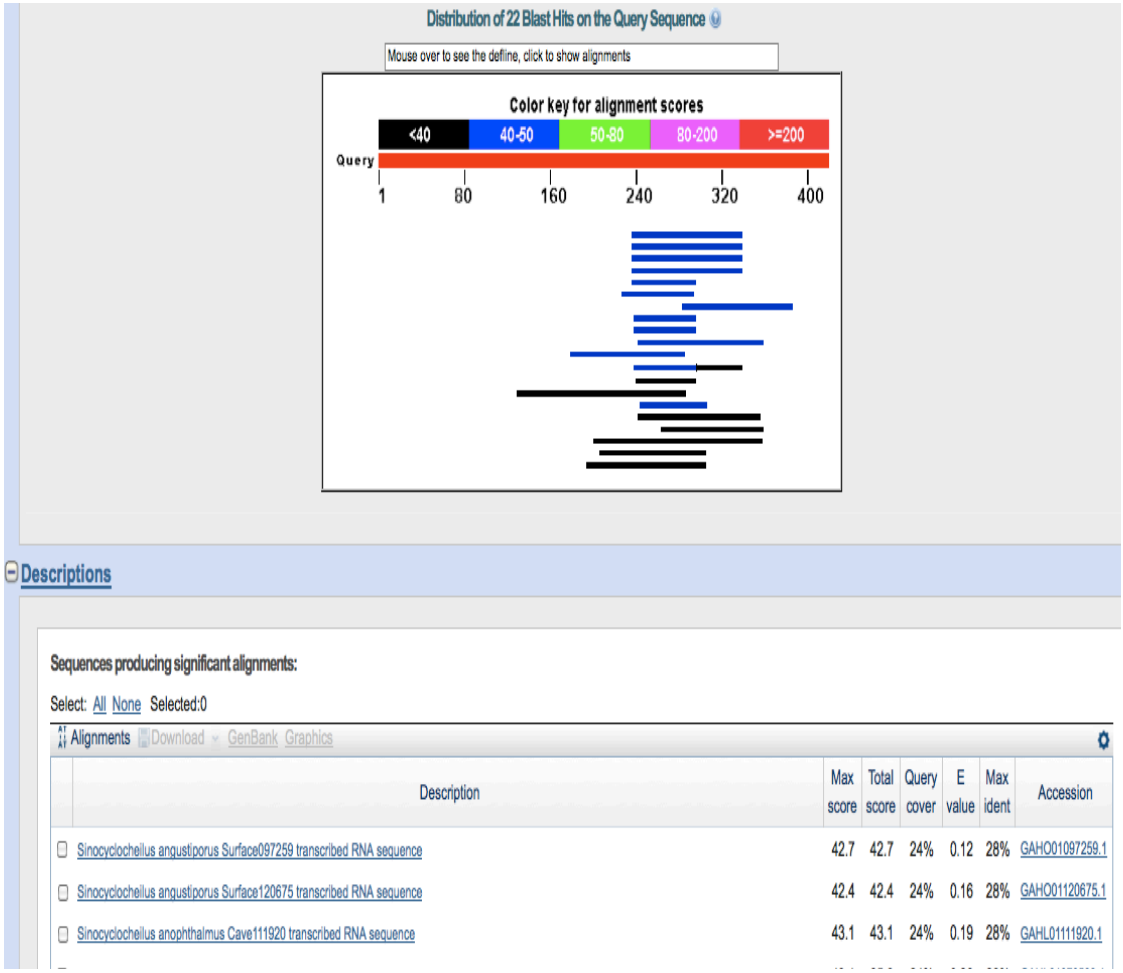
Fig. 8. Four days-old adult desert locusts were first injected with 150 ng of *dsRNase2* or *gfp* dsRNA and, six days later, 2 µg of *gfp* or *gapdh* dsRNA was orally injected, indicated as dsR2-dsgfp, dsR2-dsgapdh and dsgfp-dsgapdh, respectively. The *gapdh* knockdown in the midgut was assessed three days later with qPCR (mean ± SEM, n = 6-7).

5.3.4. Phylogenetic analysis of the DNA/RNA non-specific nuclease family

In most insects investigated, as well as in ten different crustacean species (all belonging to the class of the Malacostraca), we found sequences with a high degree of similarity to the deduced amino acid sequences of the *dsRNAses* of *S. gregaria*. In contrast, all other publicly available eukaryotic nucleotide sequence databases lack clear *dsRNase*-orthologous sequences. This is exemplified in Fig. 9, where we compared the deduced amino acid sequence of *dsRNase1* of *S. gregaria* to the translated nucleotide sequence databases of eukaryotes available in Genbank, without the sequence resources belonging to crustacean or insect species (Fig. 9A). Whereas this search did not hit upon sequences with a high degree of sequence homology, including the available crustacean sequence resources resulted in the identification of multiple sequences with a high degree of homology (Fig. 9B).

A

Eukaryota (minus insects and crustaceans)



B

Eukaryota (minus insects)

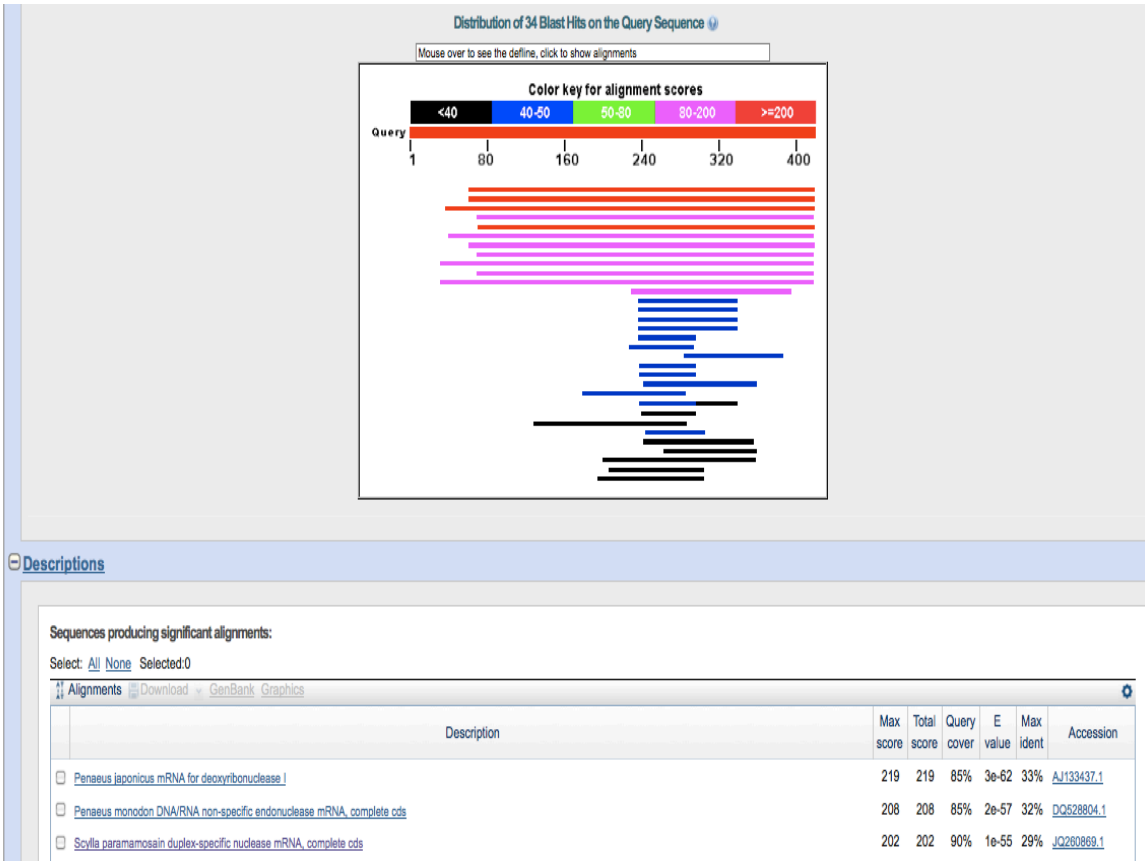


Fig. 9. Comparing the deduced amino acid sequence of the *Sg-dsRNase1* to the entire publicly available eukaryotic nucleotide database in Genbank (NCBI) (A) minus the sequences belonging to insects and crustacean species and (B) minus the sequences belonging to insect species by means of tBLASTn.

Both prokaryotes and eukaryotes possess proteins that contain a single NN-domain and belong to the DNA/RNA non-specific nucleases (Meiss *et al.*, 1999; Ikeda *et al.*, 2002). Whereas in bacteria this group of proteins are generally designated as "Non-specific Nucleases", the eukaryotic homologues are most often referred to as "Endonuclease (Endo)G" proteins. Deciphering the evolutionary history of the dsRNases and DNA/RNA non-specific nucleases suggests that the eukaryotic *dsRNase* and *endoG* sequences share a common (bacterial) origin, but cluster in two separate groups (Fig. 10). This is well bootstrap (100 replicates) supported, with a probability of 1.0.

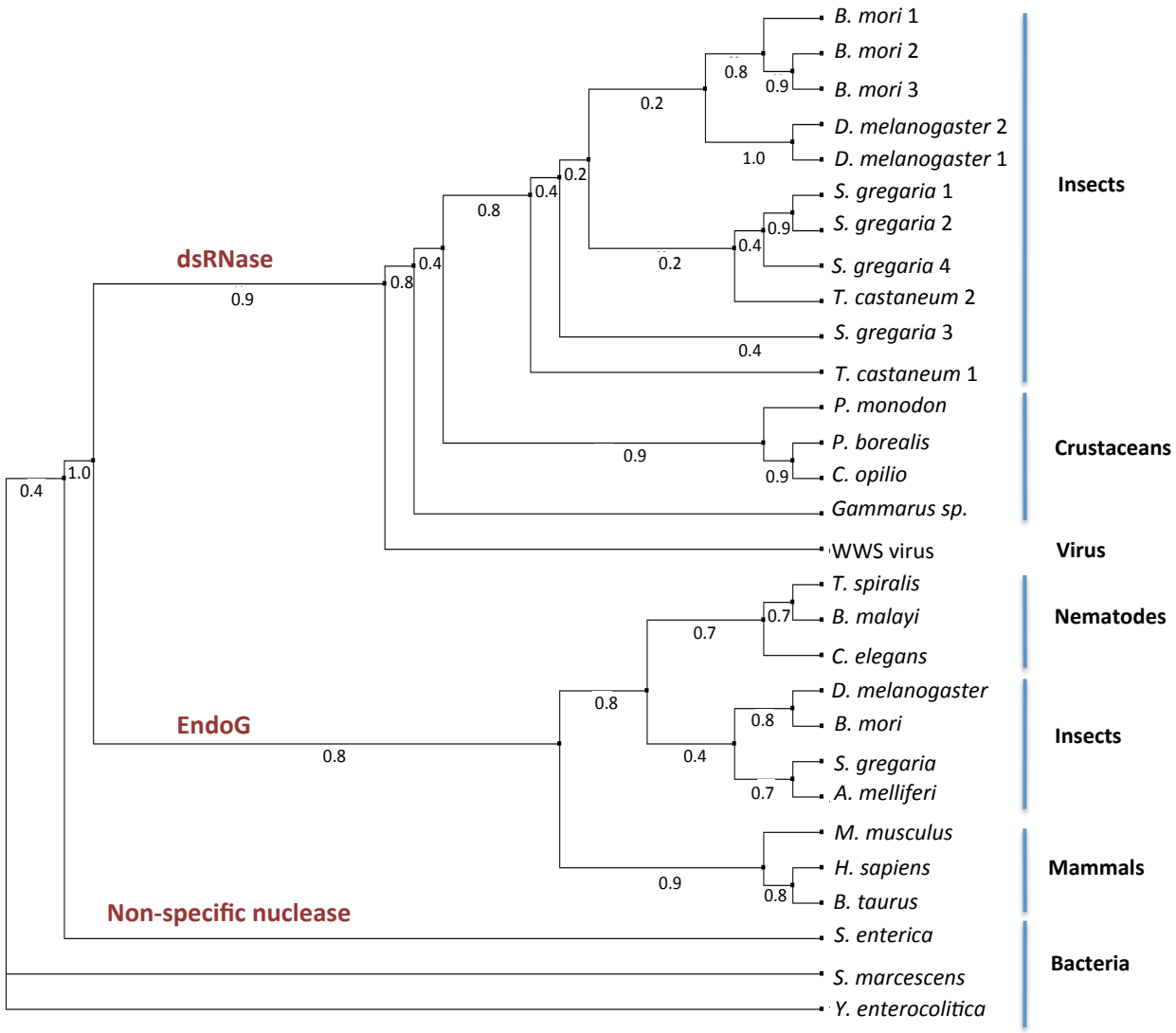


Fig. 10. A maximum-likelihood phylogenetic tree (100 bootstraps) using the most conserved region of approximately 100 amino acids between DNA/RNA Non-specific Nuclease family members belonging to insects, crustaceans, nematodes, mammals and bacteria. We used the dsRNase sequences of *Bombyx mori* (*B. mori 1* (AK383943.1), *B. mori 2* (NM_001098274.1), *B. mori 3* (XM_004922778.1)), *Drosophila melanogaster* (*D. melanogaster 1* (NM_140821.4) *and D. melanogaster 2* (NM_140819.2), *Tribolium castaneum* (*T. castaneum 1* (XM_968494.2) *and T. castaneum 2* (XM_965401.1)), *Schistocerca gregaria* (*S. gregaria 1, S. gregaria 2, S. gregaria 3 and S. gregaria 4*), *Penaeus monodon* (DQ528804.1), *Pandalus borealis* (FN554584.1), *Chionoecetes opilio* (AB542197.2), *Gammarus species* (DQ862539.1) and of the white spot syndrome (WSS) virus (AY897233.1). In addition, we used bacterial Non-specific Nuclease sequences of *Serratia marcescens* (M19495.1), *Salmonella enterica* (AY064419.1) *and Yersinia enterocolitica* (AM286415.1) and EndoG sequences of *Trichinella spiralis* (XM_003375631.1), *Brugia malayi* (XM_001892317.1), *Caenorhabditis elegans* (NM_058970.4), *D. melanogaster* (NM_140819.2), *B. mori* (XM_004925304.1), *S. gregaria*, *A. mellifera* (XM_624104.3), *Mus musculus* (NM_007931.1), *Homo sapiens* (AY892084.1) and *Bos taurus* (X72802.1).

Interestingly, we also found a *dsRNase* in the genome of the shrimp white spot syndrome (WSS) virus, which was predicted to encode a single NN-domain and a eukaryotic signal peptide (Fig. 11A). In addition, including all the publicly available crustacean dsRNases into the dataset, the WSS dsRNase was found to cluster between the bacterial and crustacean dsRNases (Fig. 11B), with a probability of 1.0 (for 100 bootstraps).

In most insect species for which genomic resources are available, multiple paralogous *dsRNAse* genes and a single *endoG* gene were found (Fig. 12). However, there are some exceptions. The genome sequence information of the beetle *T. castaneum* lacks an *endoG like* sequence. However, this is not the case for all beetles, since an *endoG* homologue was found for the beetle, *Dendroctonus ponderosae* (data not shown). Furthermore, *dsRNase* sequences seem to be absent in the publicly available sequence databases of bees and ants, while the wasp, *Nasonia vetripennis*, (which is also an *Hymenoptera*) possesses four *dsRNase* paralogues.

A

dsRNase of the WSS virus:

MNLLPIFLTTFFVAVDACSCSTICLLPDGKKQPLVFDSVLEEVVYPTDVCGPKGAGELFTGVDLLTLCIGGKNNG
GEWSGKGPCPRINNAVVERDYSLDEEDCKGFRKGFRIPGTDHFHTVFSLCWVDRDMHAKWVRNKINPGIVTD
DEDLVDSGIRTKFKYSSKIFGKGFNPRPLYSLDYQERIKILKSHFNKRTGNFFARGHLAPAGDFFLASERWATFA
LENAVPQIQNHNNGEWKDIENRARTTPGAAWAETGPIFYQHKKKEYLDKKKKYIPIPHALYKIVYDKNNKELF
RVQSDMSWK

B

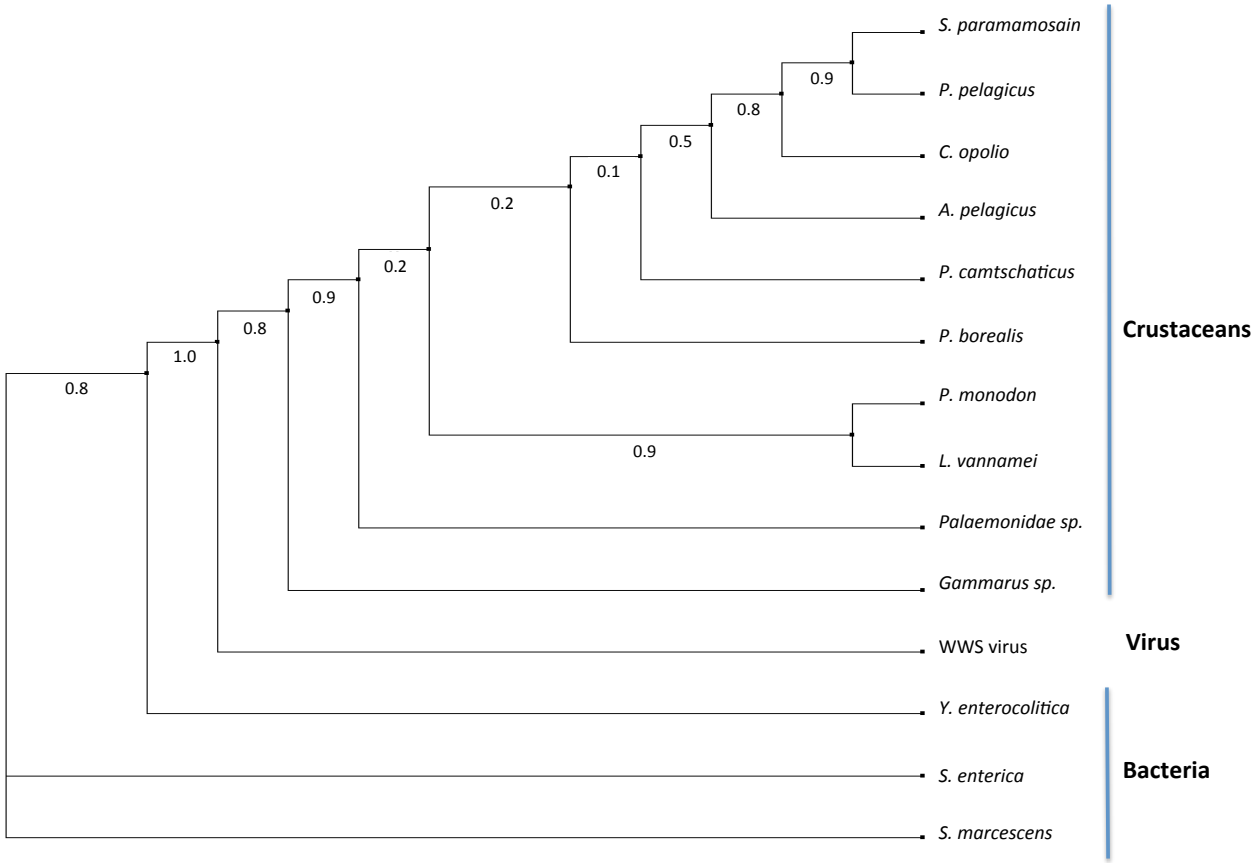


Fig. 11. (A) The deduced amino acid sequence of the white spot syndrome (WSS) virus *dsRNase*, with the NN-domains (Pfam) depicted in yellow and the eukaryotic signal peptide (SignalP) shown in green. (B) Maximum likelihood phylogenetic tree (100 bootstraps) with in the dataset the most conserved region of approximately 100 amino acids for all the available crustancean dsRNase sequences in Genbank (*Penaeus monodon* (DQ528804.1), *Pandalus borealis* (FN554584.1), *Chionoecetes opilio* (AB542197.2), *Gammarus species* (DQ862539.1), *Scylla paramamosain, Portunus pelagicus, Paralithode camtschaticus, Palaemonidae sp., Litopenaeus vannamei* and *Amphica pelagicus*), the dsRNase of the white spot syndrome (WSS) virus (AY897233.1) and of three different bacteria (*Serratia marcescens* (M19495.1), *Salmonella enterica* (AY064419.1) and *Yersinia enterocolitica* (AM286415.1))

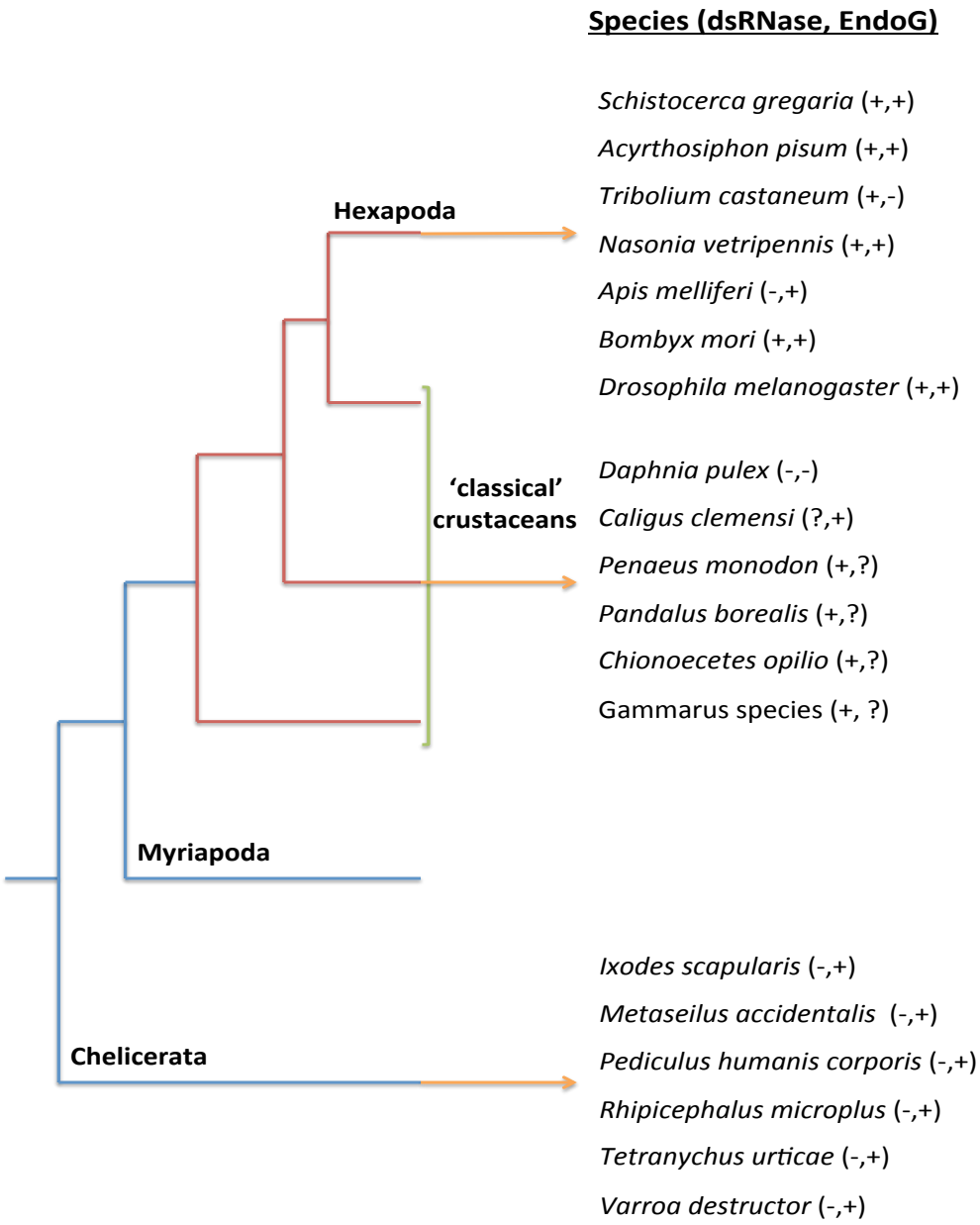


Fig. 12. *dsRNase* and *endoG* sequences present in different arthropod species. The presence of a *dsRNase* or *endoG* sequence is indicated by '+', the absence by '-' and '?' is used to depict the fact that we were unable to find a sequence in this species, but due to the lack of genome sequence information, its presence remains undetermined.

In the *S. gregaria* transcriptome database, we found an *endoG-like* transcript sequence that was predicted to encode a protein with a single NN-domain, but lacked a signal peptide sequence (Pfam) (Fig. 13A) and displays a widespread transcript tissue distribution in seven days old adult desert locusts (Fig. 13B).

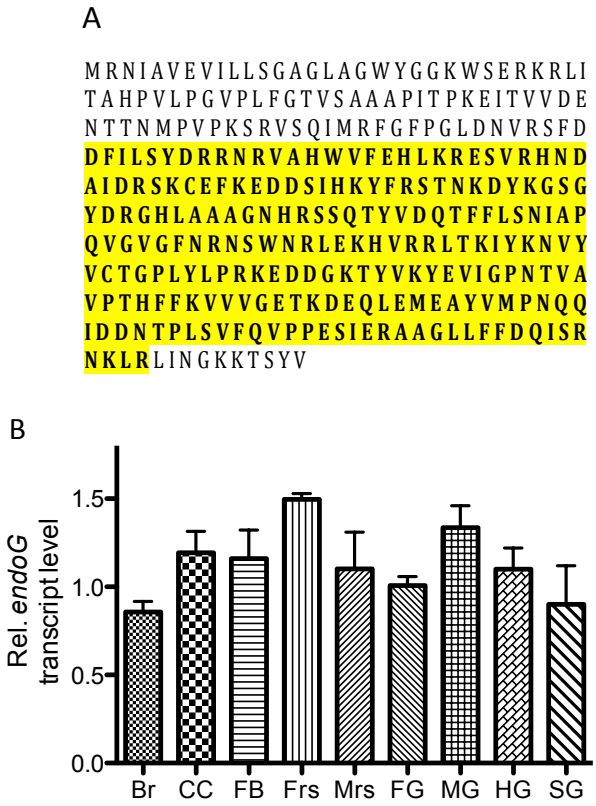


Fig. 13. (A) The deduced amino acid sequence of *endoG* of the desert locust. The predicted NN- domain (using Pfam) is depicted in yellow. (B) The *endoG* transcript profile for seven-day old adult locusts. Each bar represents the mean of two independent pools of adult males (40 and 10 animals/pool) and two independent pools of adult females (40 and 10 animals/pool). For the male and female reproductive systems only males or females were used, respectively (mean ± SEM) (Br= brain, CC= corpora cardiaca, FB= fat body, Frs= female reproductive system, Mrs= male reproductive system, FG= foregut, MG= midgut, HG= hindgut and SG= salivary glands).

5.3.5. Apo3 participates in the formation of the GMS

To identify the agent(s) that bind to dsRNA in the serum of the desert locust (§ 5.3.1), we assessed the protein content of the GMS-band by means of SDS-PAGE. Regarding the difference in the migration pattern of the GMS-band in response to the concentration of the serum (Fig. 2A), we analysed the protein content of two GMS-bands that had a clear difference in electrophoretic mobility (Fig. 14A). Determining the masses of the proteins present in the GMS-band by means of SDS-PAGE showed two protein bands with a mass of approximately 20 kDa and one protein band of approximately 70 kDa (Fig. 14B). For both GMS bands analysed, the same proteins

appear to be present. To determine the identity of the two proteins with a mass of approximately 20 kDa, we sequenced their N-terminus by means of Edman degradation. For both sequences, the N-terminal sequence corresponded to the deduced amino acid sequence of *apolipophorin* (*apo)3*, found in the *S. gregaria* transcriptome database, and therefore constitute two isoforms of Apo3. The N-terminal sequence obtained with Edman degradation is displayed in Fig. 14C.

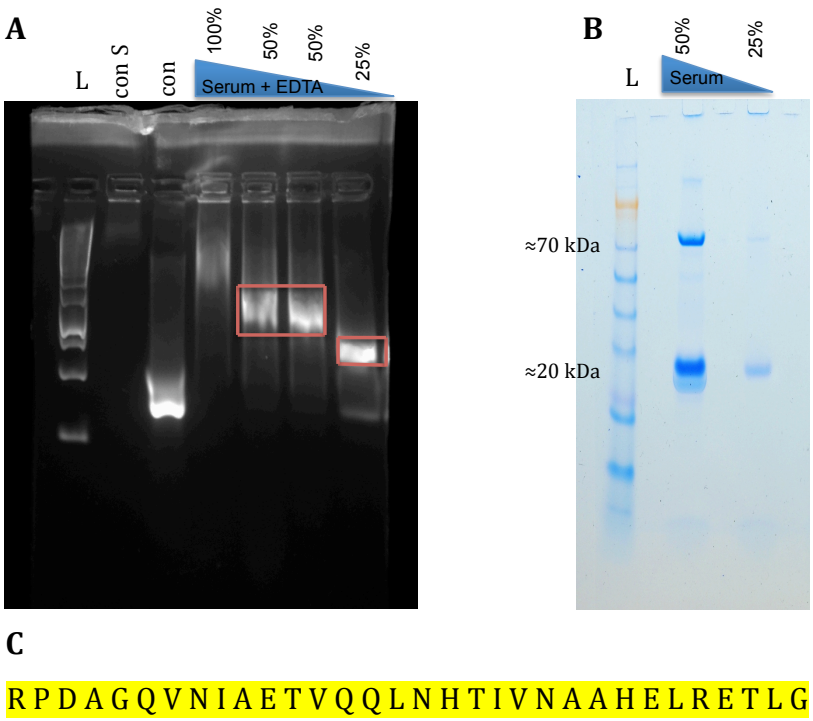


Fig. 14. (A) Incubation of dsRNA in different concentrations of serum supplemented with 0.05 M EDTA. Next, two GMS-bands with clear difference in electrophoretic mobility (indicated with red rectangles) were cut out from the 1% agarose gel (L= 1 Kb DNA ladder, con S = pure serum sample and con = dsRNA incubated in *Sg*-Ringer). (B) The protein content in these GMS-bands was purified and subsequently concentrated by means of a RotaVap lyophilisator (FTC Systems, Inc.). Eight µl of this solution was analysed by means of SDS-PAGE, prepared with NuPAGE® Bis-Tris Mini Gels (Life technologies Co.). The staining was performed with SimplyBlue™ Safe stain (Life technologies Co.) and the used ladder (L) was SeeBlue® Plus2 Pre-Stained Standard (Life technologies Co.). (C) Following the transfer of the proteins to a PVDF membrane with the cell II™ Blot module (Life technologies Co.), the proteins were stained with amido black solution and subsequently the N-terminal amino acid sequence of the two bands with a mass of approximately 20 kDa was determined by means of automated Edman degradation.

5.3.6. Lipophorins bind to dsRNA

Apo3 exists either as a water-soluble, monomeric, lipid-free protein or, especially in periods of high-energy consumption, associates with a protein-lipid complex termed lipophorin. These protein-lipid complexes consist of two other Apolipophorins, namely Apo1 and Apo2, and are considered being key-players in the transport of lipids in insects. The complete precursor for *apo1* and *apo2* (*apo1/2*) was present in the *S. gregaria* transcriptome database and the resulting proteins were predicted to have a mass of approximately 294 kDa and 76 kDa, respectively. Since we found a protein with a mass of approximately 70 kDa and a protein band that was still present in the well of the polyacrylamide gel (Fig. 14B), it would not seem surprising that the entire lipophorin complex contributes to the dsRNA-binding activity. Therefore, we purified lipophorins from the hemolymph of the desert locust by means of KBr-density gradient ultracentrifugation (Fig. 15A) and assessed their protein content with SDS-PAGE (Fig 15B). The SDS-PAGE profile of the purified lipophorins showed high similarity to this of the GMS-band. A protein band with a mass of approximately 5 kDa was also present in the lipophorins solution, yet the identity of this protein was not further investigated. After removing the K^+ and Br^- -ions from the lipophorin sample through dialysis with *S. gregaria* Ringer solution, we added 10 µl of lipophorin solution to 150 ng of dsRNA for 10 minutes, demonstrating that lipophorins can bind to dsRNA (Fig. 14C).

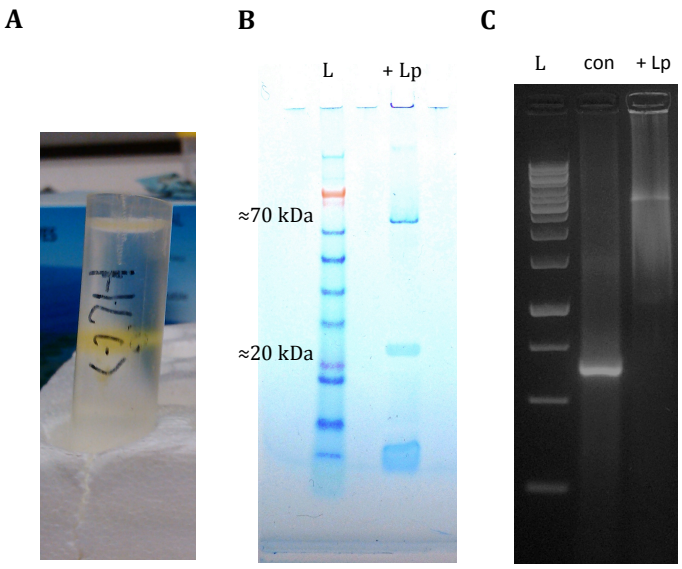


Fig. 15. (A) KBr-density gradient ultracentrifugation of hemolymph of adult desert locusts. The yellow band contains the lipophorins and was collected with a syringe and stored at -80°C. (B) The protein content of 8 µl of the lipophorin solution (+ Lp) determined by means of SDS-PAGE using the NuPAGE® Bis-Tris Mini Gels (Life technologies Co.). The proteins were stained with SimplyBlue™ Safe stain (Life technologies Co.) and the used ladder (L) was SeeBlue® Plus2 Pre-Stained Standard (Life technologies Co.). (C) Administering 10 µl of lipophorin solution (+ Lp) to 150 ng of dsRNA (for 10 minutes). Next, the dsRNA was visualised by means of 1% agarose gel electrophoresis. As a control, dsRNA was also incubated in *Sg*-Ringer (con), (L= 1 Kb DNA ladder (Fermentas)).

5.3.7. dsRNA-binding activity in the serum of other insects

Serum of the fly *Sarcophaga crassipalpis*, the cricket *Acheta domesticus* and the cockroach *Periplaneta americana* was collected. Yet, due to high levels of natural fluorescence (in the presence of GelRed and UV-light) in the serum of *S. crassipalpis* and *P. americana*, Cy3-labelled dsRNA was used to assess the dsRNA-degradation and –binding activity. Following the incubation of 100 ng of Cy3-dsRNA in different dilutions of the serum, the samples were analysed by electrophoresis and the Cy3-dyes were visualized by means of an Ettan DIGE imager (GE healthcare). To ensure the specificity of the Cy3-signal, GelRed was not added to the agarose gel. Our data demonstrate that in *S. crassipalpis*, *A. domesticus* and *P. americana* part of the Cy3-dsRNA was hindered to migrate to its full extend (Fig. 16).

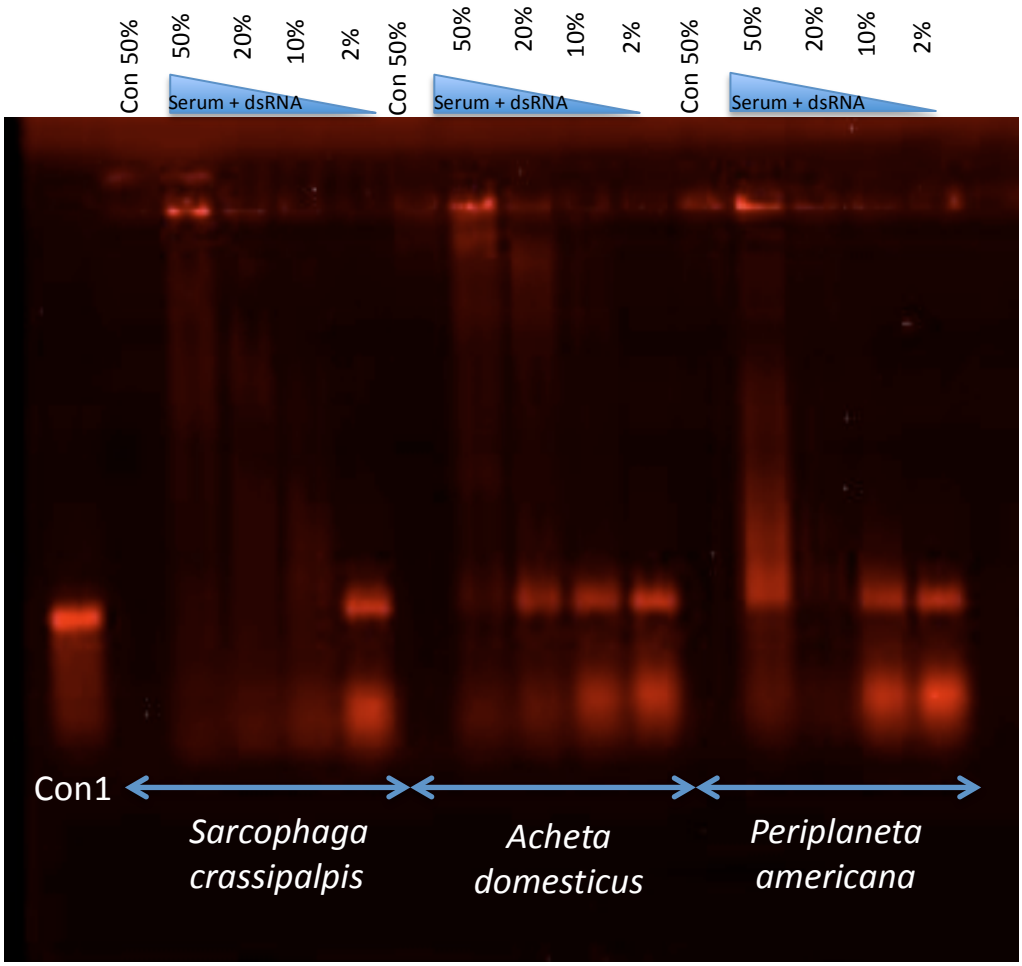


Fig. 16. Incubation and separation of 100 ng of Cy3-dsRNA in different concentrations of serum of *S. crassipalpis*, *A. domesticus* or *P. americana* for 5 minutes. The samples were then electrophorised using an agarose gel without adding GelRed (Fermentas) to the gel. The Cy3-dye was visualised by using an Ettan DIGE imager (GE healthcare) (con 50% = Cy3-dsRNA incubated in ½ diluted serum sample; con1= Cy3-dsRNA incubated in *Sg*-Ringer solution).

5.3.8. Transcript profiling of *apo1/2* and *apo3*

By means of qPCR, we assessed the transcript profile of *apo1/2* and *apo3* (Fig. 17). Whereas, the highest *apo1/2* and *apo3* transcript levels were reported in the fat body, more limited expression levels were also found in the testes, the corpora cardiaca and the salivary glands. In addition, the central nervous tissues investigated (brain, thoracic ganglia and suboesophageal ganglion) possessed limited *apo3* transcript levels. These experimental data contribute to the design of further experiments. For instance, to reduce the Apolipophorin levels in the hemolymph by means of RNAi, our data suggest that the knockdown of *apos* should be assessed in the fat body.

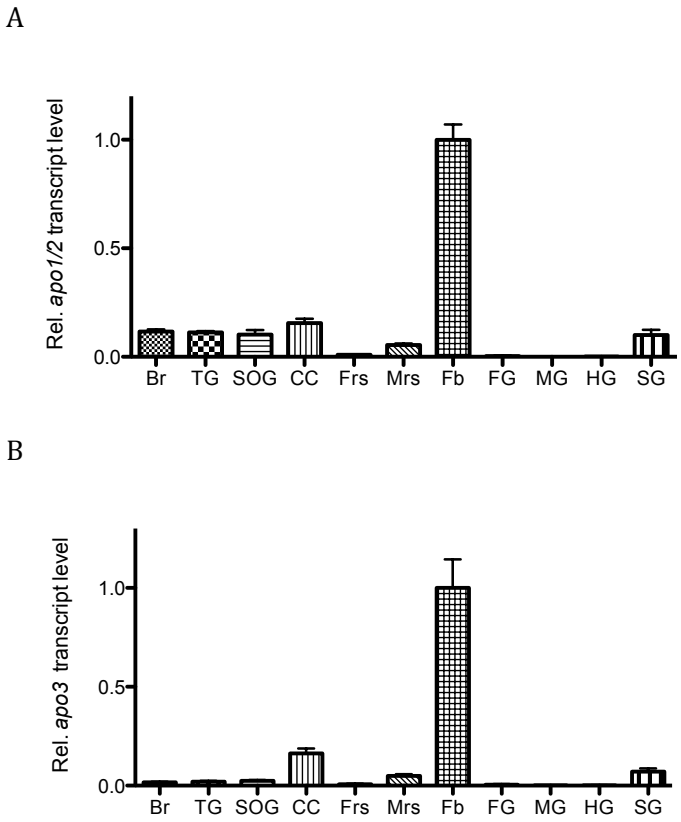


Fig. 17. Transcript profile for (A) *apo1/2* and (B) *apo3* in seven days old adult desert locusts. Each bar represents the mean of two independent pools of adult males (40 and 10 animals/pool) and three independent pools of adult females (40 and 10 animals/pool). For the male and female reproductive systems only males or females were used, respectively (mean ± SEM), (Br= brain, TG= thoracic ganglia, SOG= suboesophageal ganglion, CC= corpora cardiaca, Frs= female reproductive system, Mrs= male reproductive system, Fb= fat body, FG= foregut, MG= midgut, HG= hindgut and SG= salivary glands).

5.3.9. Silencing of *apolipohorins* and the possible effect on the RNAi-efficiency

The importance of lipophorins in RNAi was assessed with an 'RNAi on RNAi' approach. Therefore, the locusts were injected with 150 ng of dsRNA of *apo1/2*, *apo3* or *gfp.* Yet, six days after the dsRNA-injection, the knockdown efficiency of *apo1/2* and *apo3* was limited. In order to enhance the knockdown efficiency, we injected 5th larval instar locust with 300 ng of *apo1/2*, *apo3* or *gfp* dsRNA and repeated this dsRNA-treatment after their final moult. Six days later, a robust knockdown for *apo1/2* and *apo3* was reported in the fat body (Fig. 18A and 18B).

Next, six days after the second injection of *apo1/2* or *apo3*, the locusts were injected with 150 ng of *gapdh* dsRNA. The potency of the *gapdh* knockdown in the midgut

tissue was used as a marker for the RNAi-potency, 16 hours after injection of ds*gapdh*. As control, locusts that were injected twice with ds*gfp* were, 6 days later, injected with 150 ng ds*gapdh.* A second control group that was treated three times with *gfp* dsRNA was also taken into account. Since in this group the *gapdh* gene expression was not silenced, these locusts were used to determine the normal physiological *gapdh* expression levels. The workflow of the experiment is summarized in Fig. 19. Our data demonstrate that silencing *apo1/2* or *apo3* had no significant effect on the knockdown of *gapdh* in the midgut (Fig. 18C).

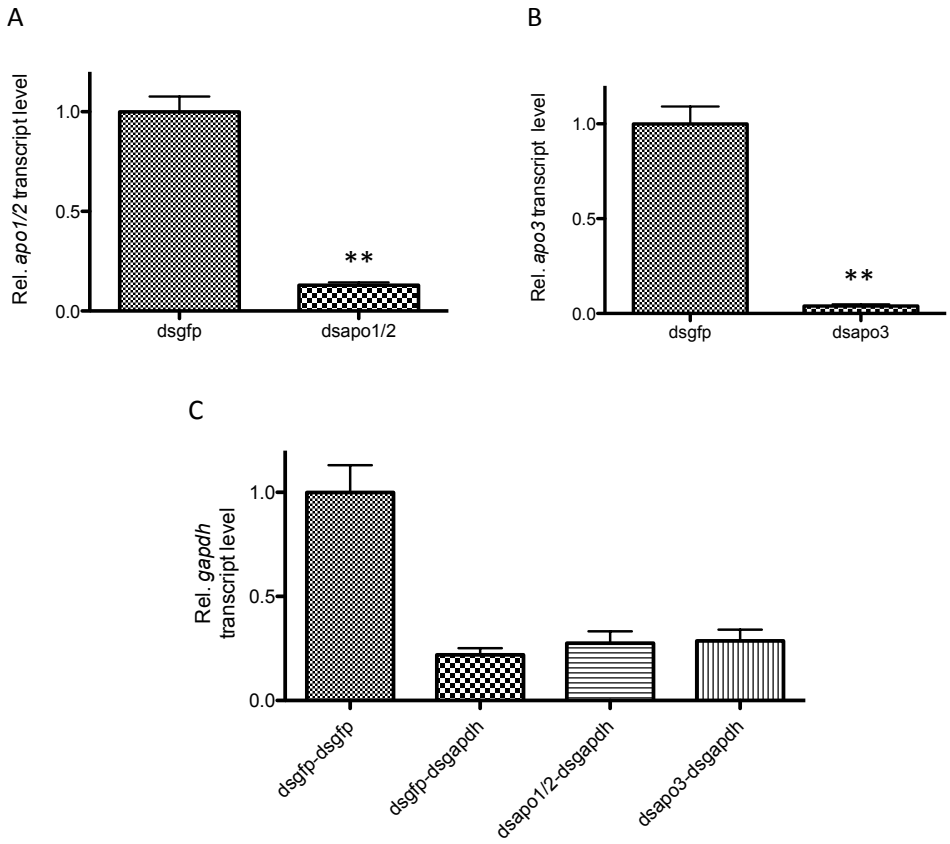


Fig. 18. RNAi on RNAi for *apo1/2* and *apo3*. Fifth larval instar locusts were injected with with 300 ng of (A) ds*apo1/2*, (B) ds*apo3* or ds*gfp*. In their adult stage, these locusts were treated with a second dsRNA-injection and, six days later, the knockdown was assessed in the fat body by means of qPCR (n = 5-6; ** = $p < 0.01$). (C) Next, these locusts were also injected with 150 ng of dsRNA for *gapdh* or *gfp*. Sixteen hours later, the knockdown of *gapdh* was determined in the midgut by using qPCR. Locusts that were three times injected with ds*gfp* are indicated as dsgfp-dsgfp. Locusts that were twice injected with ds*gfp*, ds *apo1/2* or ds*apo3* and once with ds*gapdh* are indicated as dsgfp-dsgapdh, dsapo1/2-dsgapdh and dsapo3-dsgapdh, respectively (mean ± SEM, n = 7-10).

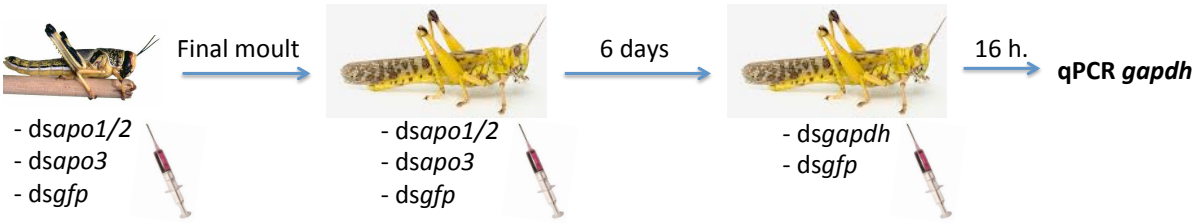


Fig. 19. The workflow of the 'RNAi on RNAi' approach for *apo1/2* and *apo3*. We injected 5th larval instar locust with 300 ng of *apo1/2*, *apo3* or *gfp* and repeated this dsRNA-treatment after their final moult. Six days later, these locusts were injected with ds*gapdh* or ds*gfp*. After 16 hours the knockdown of the *apolipophorins* and *gapdh* was assessed by means of qPCR.

5.3.10. Lipophorins do not seem to protect the dsRNA against degradation

Next, we tested whether lipophorin-binding to dsRNA may protect the dsRNA against enzymatic degradation. Therefore, we added 10 µl of the lipophorin solution to 150 ng of dsRNA and waited for 5 minutes. Then, we added 5 µl of 5-times diluted midgut juice to this sample. The samples were further diluted to improve the sensitivity of the assay. As a control, 150 ng of dsRNA was incubated in *Sg*-Ringer solution and 5 minutes later 5 µl midgut juice was added to digest dsRNA for 3 minutes. In addition, 150 ng of dsRNA was also incubated for ten minutes in 10 µl *S. gregaria* Ringer solution, representing undigested dsRNA. Next, we dissociated the nucleoprotein complex by means of phenol-chloroform extraction, pelleted the dsRNA with ethanol precipitation and assessed the integrity of the dsRNA with 1% agarose gel electrophoresis. Following the binding of lipophorins to dsRNA, protection against nucleases in the midgut juice was not observed (Fig. 20A). Yet, it remained possible that the lipases and proteases in the midgut juice destroyed the lipohorin and so its putative protective role. Therefore we repeated this experiment, but as source for dsRNase-activity we used (5-times diluted) serum instead of midgut juice. However, protection of dsRNA against enzymatic degradation of the serum was not observed (Fig 20B).

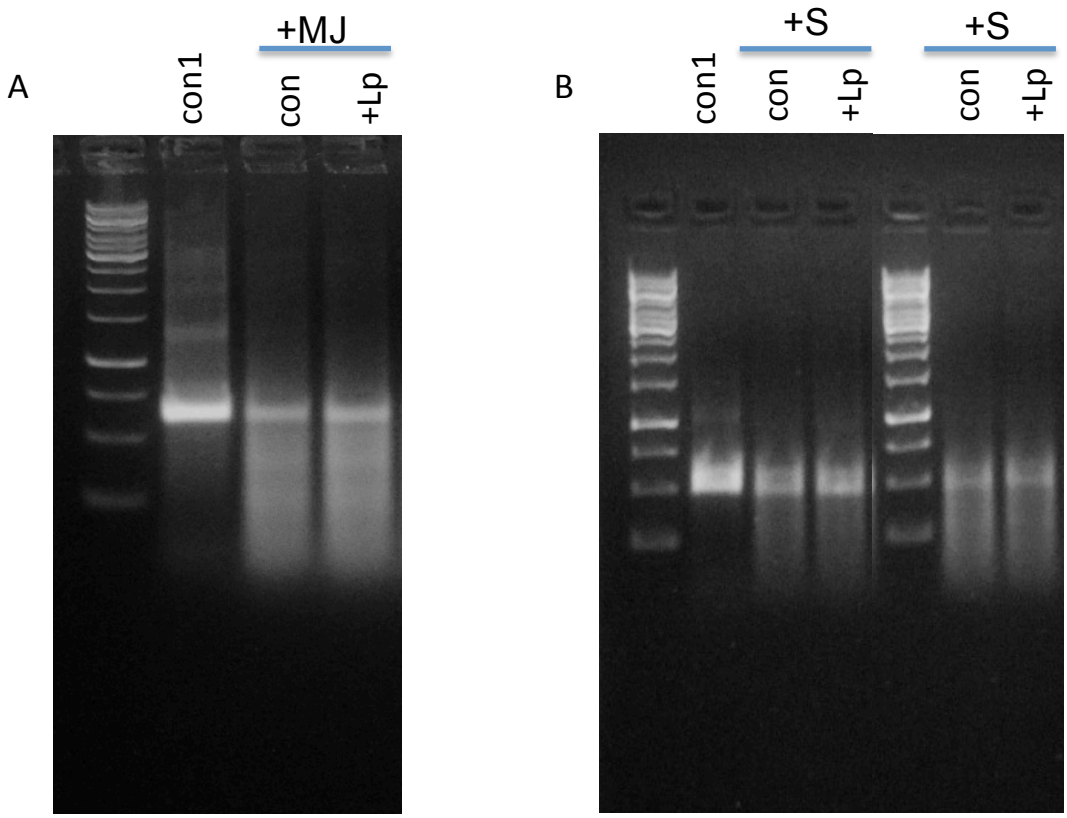


Fig. 20. (A) 150 ng dsRNA was incubated in 10 µl *Sg*-Ringer (con) or in 10 µl Lipophorin solution (+Lp) and 3 minutes later, 5 µl of 5 times diluted midgut juice (+MJ) was added. The nucleoprotein complexes were dissociated by means of phenol-chloroform extraction and the samples were analysed with 1% agarose gel electrophoresis (con1 = dsRNA incubated for 10 minutes in 10 µl *Sg*-Ringer). (B) 150 ng of dsRNA incubated in 10 µl *Sg*-Ringer (con) or in 10 µl Lipophorin solution (+Lp) and 3 or 5 minutes later we added 5 µl of 5 times diluted serum sample (+S). Following the dissociation of the nucleoprotein complexes with phenol-chloroform extraction, the integrity of the dsRNA was assessed by means of 1% agarose gel electrophoresis.

5.4. Discussion

5.4.1. Degradation of dsRNA in midgut juice and serum

In this study, we have assessed the persistence of dsRNA in midgut juice and serum of the desert locust, and reflected on the effect of dsRNA-degradation on the success of RNAi. In particular, it was hypothesized that rapid degradation of dsRNA could be an explanation for the deficient RNAi-response of the desert locust to orally delivered dsRNA (Chapter 3). Therefore, we developed an *ex-vivo* dsRNA-degradation assay.

Here, dsRNA was incubated in midgut juice or serum. Later on, the integrity of the dsRNA was analysed by means of agarose gel electrophoresis. Surprisingly, rapid enzymatic degradation of dsRNA was not only reported in midgut juice, but also in serum. Apparantly, the dsRNA-degradation was more efficient in the midgut juice, since the midgut juice was dissolved in *Sg*-Ringer solution (and therefore diluted several times), while pure serum was analysed and still residual dsRNA-levels remained present in the serum but not in the gut juice (Fig. 1). In addition, since administering EDTA to the reaction could inhibit the dsRNase-activity, the nucleases in the midgut juice and serum appear to require cationic cofactors for their dsRNase-activity (Fig. 1), which is in full agreement with the fact that the *B. mori* alkaline nuclease requires Mg^{2+} for its activity (Arimatsu *et al.*, 2007).

It is remarkable that injection of dsRNA can induce potent gene silencing effects in the desert locust, while the majority of the dsRNA seems to be degraded in the serum. One possible explanation could reside in the fact that only very small quantities of dsRNA are needed to mediate RNAi in the desert locust, as demonstrated in Chapter 3, where injection of pg dsRNA per mg tissue was sufficient to induce potent gene silencing in adult locusts. Therefore, it seems plausible to consider that only low dsRNA-quantities will sustain in the hemolymph, while the excess of dsRNA will be enzymatically digested.

Garbutt *et al.* (2013) developed an assay to assess the *in vivo* dsRNA-persistency in the body cavity of the tobacco hornworm, *M. sexta*, by means of qPCR. Therefore, dsRNA of *gfp* was injected, and the remaining ds*gfp* levels (at several time points) were assessed by means of qPCR. Although qPCR is typically used for the detection of single stranded RNA, they demonstrated that it could also be used to detect dsRNA. Following the injection of high dsRNA-quantities (4 µg), they calculated the average *in vivo* half-life of the *gfp* dsRNA in the hemolumph of *M. sexta* as 27 minutes.

In contrast to the situation in *M. sexta*, *in vitro* assays illustrated that in the serum of the german cockroach, *B. germanica*, dsRNA persists much longer (Garbutt *et al.*, 2013). Therefore, the authors proposed that the success of sysRNAi in this cockroach could be due to the high persistence of the dsRNA in the serum. In our study, we assessed the integrity of dsRNA in the serum of the American cockroach, *P. americana* and, although it remains difficult to draw final conclusions, very limited dsRNA-degradation was observed (Fig. 16). Yet, based on the situation in the desert locust,

persistence of high dsRNA-levels in the hemolymph does not seem to be a necessary prerequisite for susceptibility to RNAi. For robust RNAi, the locust must possess a highly sensitive cell-autonomous RNAi-response or a (unidentified) dsRNA-amplification mechanism.

Following the incubation of 150 ng of dsRNA in 10 µl (undiluted) serum for 5 minutes, we were unable to detect unbound-dsRNA. The dsRNA was either degraded or bound to lipophorins (Fig. 1 and 2). Keeping in mind the fact that the average total blood volume of an adult desert locust is approximately 200 µl (Lee, 1961), the dsRNA-level in the *ex-vivo* experiments corresponds to the injection of 3 µg of dsRNA per locust. Since, dsRNA is one of the most important viral-associated molecular patterns and could represent the genome of a dsRNA-virus, it might not seem surprising that the dsRNA-titers in the hemolymph are tightly regulated. For instance, to avoid a conflict of interest, the insect shouldn't sustain higher dsRNA-levels than needed to activate the anti-viral immune response, certainly when knowing that lipophorins are also crucial components in the transfer of energy and can recognize other pathogens, such as bacteria and fungi (Dettloff *et al.*, 2001; Ma *et al.*, 2006). In addition, the uncontrolled cellular delivery of (potentially viral genomic) dsRNA could trigger viral infection. Therefore, maintaining control over the (entire) dsRNA-titer in the hemolymph is of particular interest.

Whereas inhibition of the RNase A family enzymes prevented the degradation of siRNAs in human blood (Haupenthal *et al.*, 2006), to date, the identity of the dsRNA-digesting nucleases in the serum of insects remains undetermined. On the other hand, we have identified a critical role for the *Sg*-dsRNase family (especially for dsRNase2) in the degradation of dsRNA in the midgut juice of the desert locust (Fig. 7), which is in full agreement with the previously published dsRNase-activity of the related *alkaline nuclease* in the midgut juice of *B. mori* (Arimatsu *et al.*, 2007). Moreover, the *Bm-alkaline nuclease* is expressed specifically by the middle and posterior midgut (Arimatsu *et al.*, 2007). In accordance to these findings, in *S. gregaria*, we identified four *Sg-dsRNAses* that are specifically expressed by the midgut (Fig. 4).

Silencing of the dsRNase2-activity in the midgut juice by means of RNAi could not render the locusts sensitive towards orally delivered dsRNA (Fig. 8). At present, the exact cause of the observed deficiency of RNAi upon oral delivery of dsRNA is not fully clear. For instance, the inhibition of the dsRNase-activity in the midgut might

have been insufficient, dsRNA-degradation could also occur in other compartments of the digestive tube or the locusts might lack the necessary mechanisms to transport the dsRNA from the gut lumen into the body.

Also in other insects, we found multiple paralogous *dsRNase* genes (Fig. 10). We obtained evidence for the involvement of *Sg-dsRNase2* in the digestion of dsRNA in the midgut (Fig. 7). However, since silencing individual *dsRNases* was challenging (Fig. 6), a possible role for *dsRNase1* in degrading dsRNA cannot be excluded. Furthermore, although only a clear effect for dsRNase2 was observed in the *ex-vivo* assay (short duration), this does not rule out a possible contributing role for the other *dsRNase* genes *in vivo* (where the exposure of dsRNA to gut juice is much longer). Further research is needed to elucidate the role of the different paralogous *dsRNases* of *S. gregaria*. One possible explanation could reside in different substrate specificity of the four *dsRNases*, such as higher affinity for ssRNA or DNA.

Remarkably, *dsRNase* homologues were exclusively found in insects and crustaceans. Nonetheless, phylogenetic analyses demonstrated that the *dsRNases* belong to the DNA/RNA non-specific nuclease superfamily and that the EndoG and the dsRNase family share a common bacterial origin, yet cluster in two different groups (Fig. 10).

Regarding the origin of the dsRNase-family in *Crustacea*, gene duplication as a source for new genetic material upon which natural selection can act might not seem likely, since higher homology with the bacterial Non-specific Nuclease than with the eukaryotic EndoG-family was found. On the other hand, the presence of a *dsRNase* gene in the genome of the WSS virus (which is in accordance with the previously published work of Witteveld *et al*., 2001) and its close relationship with the dsRNase of *Gammarus*, which is a natural host of the WSS virus, suggests that the *dsRNase* gene was subjected to horizontal transfer between the WSS virus and its crustacean host. In addition, including all the publicly available dsRNase sequences of crustaceans in the data set, suggested their evolutionary origin to reside in the WSS virus. Nonetheless, it is important to keep in mind that only limited genomic resources are available for crustaceans. Therefore, although several independent lines of evidence suggest a viral origin for the *dsRNase* family, further research is needed to draw final conclusions.

Whereas the bacterial Non-specific Nucleases seem to cleave extracellular nucleic acids as a source of nucleotides and phosphate (Rangarajan and Shankar, 2001),

EndoG proteins are typically located in the mitochondria where they will assist mitochondrial DNA replication and, following their release into the cytoplasm and translocation to the nuclei, contribute to genomic DNA-degradation during apoptosis (Low, 2003). In line with this, the *Sg*-EndoG sequence was predicted to lack a signal sequence. Moreover, the widespread tissue distribution of *Sg-endoG* suggests a different function for *Sg-endoG* than the *Sg-dsRNases* (Fig. 13). Interestingly, it has been described that an isoform of the *Bm-dsRNase* can be active in the metabolism of nucleic acids in the cytoplasm (Liu *et al.*, 2012b). Moreover, silkworms infected with the cytoplasmic polyhedrosis virus showed induced expression levels of the *Bm-dsRNase* (Wu *et al.*, 2009). Considering that viral genomes are protected by the protein capsid in the extracellular medium, the combined activity of proteases and dsRNases could digest the viral capsid and genomic information. Furthermore, since systemic spread of dsRNA is required for effective anti-viral immunity, as this was demonstrated in the fruit fly (Saleh *et al.*, 2009), the secreted dsRNase in the WSS virus might reflect a viral RNAi suppressor protein that acts through preventing the systemic spread of the RNAi-signal. Taken together, whereas a role for the dsRNases in digestion of nucleic acids as source for nucleotides and phosphates cannot be precluded, a role in antiviral immunity should also be considered. Nevertheless, knocking down vital genes with RNAi can severely affect the viability of the insect. Therefore, *in planta* production of dsRNAs that target transcripts of the phytophagous insect could protect plants against their predators. Thus, although no experimental evidence is available, it remains possible that plant-insect co-evolution has driven insects to degrade dsRNA in the alimentary tract, preventing plant-directed RNAi-responses.

Remarkably, dsRNases were found in all insects investigated with the exception of bees and ants. Since miRNAs and siRNAs have been proposed to mediate intercellular and even interspecies communication (Whangbo and Hunter, 2008), it is remarkable that these social insects lack dsRNases. Moreover, oral delivery of dsRNA has been proven successful to induce RNAi-effects in bees. Yet, further research is needed to draw final conclusions on this topic.

5.4.2. Lipophorins in the hemolymph bind to dsRNA

In this chapter, we also report on the fact that lipophorins of *S. gregaria*, which are present in the hemolymph but not the gut lumen, bind to dsRNA (Fig. 15). In this respect, a role for the lipophorins in promoting RNAi might also be considered as a plausible explanation for the discrepant RNAi-susceptibility upon injection and oral delivery of dsRNA. Lipophorins typically act in the delivery of energy to target tissues. Yet, in the presence of bacteria and fungi, they have also been reported to stimulate cellular and humoral immune responses in the moth *Galleria mellonella* (Gotz *et al.*, 1997; Dettloff *et al.*, 2001; Whitten *et al.*, 2004; Ma *et al.*, 2006) and the mosquito *A. aegyptus* (Cheon *et al.*, 2006). Our study brings in a possible role in the detection of viral molecular patterns, in particular of dsRNA. Interestingly, where the vertebrate homologues, which are termed lipoproteins, detect bacterial and fungal pathogens, they have also been implicated in the detection of a wide range of viral particles (Singh *et al.*, 1999). Furthermore, studies have illustrated that the vertebrate lipoproteins can bind DNA- and RNA-fragments, and promote the *in vitro* and *in vivo* transfection of plasmid DNA to different tissues in rats (Guevara *et al.*, 1999; Guevara *et al.*, 2010). In this context, a role for the *S. gregaria* lipophorins in the delivery of dsRNA to the target cells might not seem illogical. In the next chapter, we will further investigate the possible role of lipophorins in the cellular delivery of dsRNA.

Our data also suggest that the dsRNA-binding activity by lipophorins is conserved throughout Insecta, since a previous study demonstrated that the lipophorins of the silkmoth *B. mori* also bind to dsRNA (Sakashita *et al.*, 2009) and since incubation of dsRNA in serum of the fly *S. crassipalpis*, the cricket *A. domesticus* and the cockroach *P. americana* also resulted in the formation of a GMS (Fig. 16). Yet, further research is needed to draw final conclusions. Taken together, lipoproteins in vertebrates and invertebrates appear to play a central role in the non-specific detection of foreign body particles, including bacterial, fungal and viral derived molecular patterns.

In our study, silencing of *apo1/2* and *apo3* did not generate detectable effects on the RNAi-efficiency (Fig. 18). Yet, as illustrated in Fig. 2, a clear GMS-band could still be observed in hemolymph samples that were diluted 10 to 50 times. Thus, an excess of lipophorins seems to be present in the hemolymph, yet binding to dsRNA is (partially) prevented by the action of the dsRNA-degrading enzymes. In this context, the concentration of lipophorins in the hemolymph might not be the determinant

factor for the quantity of dsRNA bound by lipophorins. Therefore, although silencing the apos did not result in a clear RNAi-phenotype, a role in RNAi remains plausible. Furthermore, lipophorins did not seem to protect the dsRNA against degradation in the serum (Fig. 20). Yet, further research is needed to make final conclusions. For instance, the lipophorins present in the serum might already protect part of the dsRNA. Therefore, for future perspectives, it might be of interest to compare the dsRNase-activity between lipophorin-free and normal serum.

Chapter 6

Double stranded RNA uptake mechanisms in *Schistocerca gregaria*

Chapter 6: Double stranded RNA uptake mechanisms in *Schistocerca gregaria*

6.1. Introduction

RNA interference (RNAi) is a mechanism of sequence specific gene regulation triggered by double-stranded (ds)RNA (Fire *et al.*, 1998). While RNAi is known to be involved in anti-viral immunity (Waterhouse *et al.*, 2001; Zambon *et al.*, 2006), genome maintenance (Lippman and Martienssen, 2004) and regulation of endogenous gene expression (Hutvagner and Zamore, 2002), it has also become a widely used tool to knock down and analyse the function of genes in eukaryotes (Hardy *et al.*, 2010). Several recent studies have shown that RNAi may also contribute to strategies for selectively controlling agricultural pests, including a number of insect species. However, a major challenge in exploiting the RNAi-technology remains introduction of dsRNA into the cells (Perrimon *et al.*, 2010; Yu *et al.*, 2013).

Many organisms display RNAi following injection or oral delivery of dsRNA. This phenomenon, in which RNAi is established in tissues distant from the site of dsRNA-administration, is referred to as systemic (sys)RNAi. Following a screen with mutant *C. elegans* worms, the transmembrane channel protein Systemic RNA Interference Deficient-1 (SID1) was identified as a key player in the cellular uptake of dsRNA (Winston *et al.*, 2002; Duxbury *et al.*, 2005). Expression of the *Ce-sid1* sequence in *Drosophila* Schneider (S)2 cells, which lack a *sid1* homologous sequence, significantly enhanced the sysRNAi-response (Feinberg and Hunter, 2003). In addition, homologous sequences of *sid1* in fish and mammals have been shown to mediate dsRNA-import (Duxbury *et al.*, 2005; Ren *et al.*, 2011; Elhassan *et al.*, 2012). The genomes of most insects, with the exception of dipterans, also possess genes related to *Ce-sid1*. Yet, their definite functional role in RNAi remains unclear. For example, *T. castaneum* and *L. migratoria* display a robust sysRNAi-response, but their *sid1 like* sequences do not seem to mediate uptake of dsRNA (Bucher *et al.*, 2002; Tomoyasu *et al.*, 2008; Luo *et al.*, 2012). In contrast, in *A. mellifera*, the involvement of *sid1 like* in the sysRNAi-response was suggested, since administration of dsRNA induced an up-regulation of the *Am-sid1 like* transcript levels (Aronstein *et al.*, 2006).

The best-studied insect model, *D. melanogaster*, as well as many lepidopterans are poorly sensitive towards sysRNAi, since injection of dsRNA into the body cavity is not

an effective method to induce RNAi (Bucher *et al.*, 2002; Miller *et al.*, 2008; Terenius *et al.*, 2011). For example, in the larval stage of *D. melanogaster*, the hemocytes (and the hemocyte-like S2 cell line) are the only cell type that can take up dsRNA (Miller *et al.*, 2008). Yet, since *sid1 like* sequences are absent in the genomes of these flies, S2 cells must use alternative dsRNA-uptake mechanisms. Two independent functional screens demonstrated that dsRNA enters S2 cells via Scavenger Receptor (SR) mediated endocytosis (Saleh *et al.*, 2006; Ulvila *et al.*, 2006). In the nematode *C. elegans*, silencing genes that are involved in endocytosis could also severaly impair the efficiency of the sysRNAi-response (Saleh *et al.*, 2006). Moreover, it has been suggested that SID-1 locates on the endosomes of *C. elegans* (McEwan *et al.*, 2007).

SRs constitute a group of structurally unrelated receptors. Whereas in vertebrates, there are 6 different classes of SRs, most insects only possess two classes (class B and C). Curiously, the *Drosophila* SR-CI functionally resembles the mammalian class A SRs, while the *Drosophila* class B SRs are similar to the mammalian class B SRs. In addition, another *Drosophila* SR, termed Eater, has been identified to recognize Gram-positive and –negative bacteria (Erturk-Hasdemir and Silverman, 2005; Kocks *et al.*, 2005). Saleh *et al.* (2006) were able to demonstrate that a combination of SRs participate in dsRNA uptake in S2 cells. In addition, using the RNAi-technique to target individual SRs, Ulvila *et al.* (2006) showed that silencing of *SR-CI* and *eater* led to a significant decrease in the internalization of dsRNA fragments, while silencing the class B SRs *crq*, *emp* and *ninaD* had no detectable effect on the uptake of dsRNA. Moreover, stable transfection of mammalian Chinese Hamster Ovary (CHO) cells with SR-CI was sufficient to markedly increase the dsRNA internalization in these cells (Ulvila *et al*., 2006). Vacuolar H-ATPase 16 (Vha16) is suggested to play an important role in the redirection of exogenous dsRNA from the vesicular location of the standard endocytotic uptake route into the cytoplasm, where the RNAi-machinery is localized (Saleh *et al.*, 2006).

In the previous chapter, it was demonstrated that lipophorins can bind to dsRNA in the hemolymph of the desert locust. Studies have shown that vertebrate lipoproteins can also bind to DNA and RNA, and promote *in vitro* and *in vivo* cellular-delivery of plasmid DNA (Guevara *et al.*, 1999; Guevara *et al.*, 2010). Therefore, it might not seem illogical that lipophorins could also promote the cellular delivery of dsRNA in *S. gregaria*. Currently, there are two known lipophorin-based cell entry routes; low-

density lipophorins can tightly bind to the lipophorin receptor (LpR), while high density and modified (oxidized or acetylated) lipophorins are recognized and internalized by SRs. Studies have demonstrated that, beside lipoproteins, SR ligands include polyribonucleotides, including polyinosine (Poly(I)) and polyguanosine (Poly(G)), polysaccharides, such as dextran sulphate (DS) and anionic phospholipids, including phosphatidylserine. All the known scavenger receptor ligands are polyanionic macromolecules or macromolecular complexes. Yet, many other polyanions are not ligands (reviewed in Krieger and Herz, 1994). The exact determinants that confer binding upon ligand are not fully understood. Nonetheless, progress has been made in defining the structures underlying specificity for oligonucleotides. To bind tightly to SRs, the nucleic acids should form a base-quartet-stabilized four-stranded helix (quadruplex), a conformational requirement that accounts for Poly(G) and Poly(I). On the other hand polyadenosine (Poly(A)) and polycytosine (Poly(C)) do not form this structure and will not bind to SRs (Pearson *et al.*, 1993). The underlying specificity for polysaccharides, where in contrast to DS and fucoidin, heparin and chondroitin sulphate (CS) are unable to bind SRs remains largely undetermined (Krieger and Herz, 1994). In *Drosophila* L2 cells, SR specificity was also demonstrated, showing that addition of 400 µg/ml poly(I) or DS to the medium could effectively block > 85% of the SR-mediated AcLDL uptake, while administration of their (non-binding) counterpart could not (Abrams *et al.*, 1992; Pearson *et al*., 1995). Moreover, administration of 250 µg/ml Poly(I) to the medium of *Drosophila* S2 cells could markedly reduce the dsRNA-uptake mediated by scavenger receptors (Saleh *et al.*, 2006).

Both LpR and SRs internalize the lipophorins via clathrin-dependent endocytosis. Therefore, in order to assess the involvement of lipophorins in the delivery of dsRNA to the RNAi-machinery in *S. gregaria*, we assessed in this chapter the possible involvement of clathrin-dependent endocytosis, SRs and the LpR in sysRNAi. Moreover, given the importance of SID1 in dsRNA-uptake in the nematode *C. elegans*, we have also investigated whether *Sg*-SID1 like mediates cellular uptake of dsRNA in the desert locust.

6.2. Materials and Methods

6.2.1. Sequence information

Transcript sequence information of *S. gregaria vha16* and *clathrin heavy chain* (*clath*) was retrieved from the annotated *S. gregraria* EST-database (Badisco *et al.*, 2011a), while the *lpr*-sequence was retrieved from the more recently available *S. gregaria* transcriptome database. To verify the sequences, the DNA fragments were cloned into the pCR®4-TOPO® vector by means of the TOPO TA Cloning® Kit for Sequencing (Life Technologies Co.). The sequences of the inserted DNA fragments were determined using the ABI PRISM BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems). Their identity was confirmed by reciprocal tBLASTn. In addition, following *in silico* translation into the corresponding amino acid sequences, the sequences were aligned against orthologous sequences of other insects (ClustalW) and the predicted protein domains were identified (NCBI, CD-search).

6.2.2. Synthesis of dsRNA

Double stranded RNAs for *clath* (561 bp), *vha16* (453 bp), *sid1 like* (250 bp), *lpr* (520 bp), *alpha-tubulin 1a* (*tubu*, 545 bp), *glyceraldehyde 3-phosphate dehydrogenese* (*gapdh,* 447 bp) and *green fluorescent protein* (*gfp*, 589 bp) were synthesized using the MEGAscript RNAi kit (Ambion) as described in § 2.4. The dsRNA was stored at −20 °C until further use.

6.2.3. RNA extraction and cDNA synthesis

The Lipid tissue extraction kit (Qiagen) was utilized to extract RNA, following specifications described in § 2.7. Next, equal quantities of RNA were used as template to produce cDNA. The cDNA synthesis was performed using the PrimeScript™ First strand cDNA Synthesis Kit (TaKaRa), as described in § 2.7. The resulting cDNA-samples were analysed immediately or stored at -20°C until further usage.

6.2.4. Quantitative real time PCR

The qPCR reactions were performed in accordance to the methods described in § 2.8. All data were statistically analysed by non-parametric statistics in GraphPad prism 5 (GraphPad).

6.3. Results

6.3.1. Endocytosis based uptake of the RNAi-signal

To assess the involvement of clathrin-dependent endocytosis in the sysRNAi-response in *S. gregaria*, we silenced *clath* and *vha16*. These genes were chosen based on the fact that (i) in S2 cells, knocking down these components can severely impair the uptake of dsRNA (Saleh *et al.*, 2006), (ii) they are involved in distinct steps of the clathrin-dependent endocytosis pathway, namely in the formation of coated vesicles, and lysosomal acidification, respectively, and (iii) sequence information was available in the *S. gregaria* EST-database. After injection of 150 ng of the corresponding dsRNA into the abdominal body cavity (hemocoel), silencing of these two genes in the midgut was confirmed with qPCR by comparing the transcript levels with a control group that was injected with *gfp* dsRNA (Fig. 1). The knockdown of *vha16* was assessed, 6 days after the dsRNA injection. However, silencing of *clath* gene expression induced death of the injected locusts, starting from 7 days after the dsRNA-administration. Therefore, all experiments with *clath* dsRNA were performed 4 days after the injection. At that time, there were not yet detectable defects in the behaviour of the locusts.

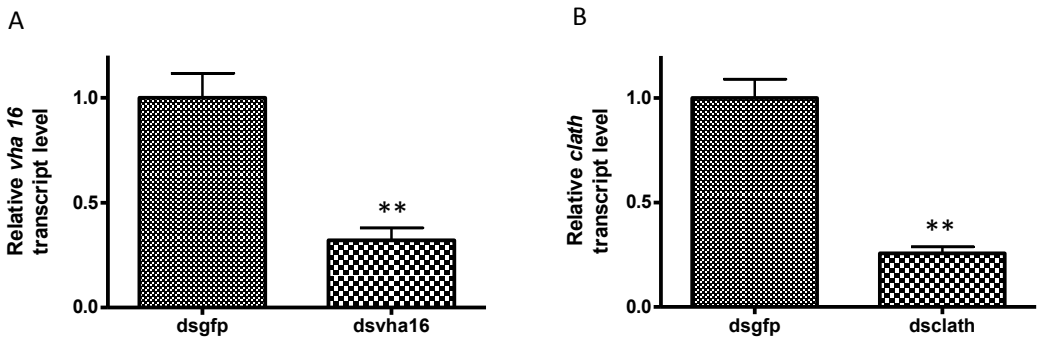


Fig. 1. Gene silencing of (A) *vha16* and (B) *clath* was verified by measuring transcript levels in the midgut. The knockdown was determined 6 days after injection of 150 ng of dsRNA of *vha16* and 4 days after injection of 150 ng of *clath* dsRNA. The transcript levels were compared to locusts injected with 150 ng of dsRNA of *gfp* (mean ± SEM; n = 6-7; **: $p < 0.005$).

Next, an 'RNAi on RNAi' approach was followed to investigate whether these endocytotic pathway components contribute to the sysRNAi-response in the RNAi-sensitive midgut tissue. First, we silenced *vha16* by injection of 150 ng of its transcript-specific dsRNA. Six days later, a second injection was performed with 150 ng of *tubu* dsRNA. The potency of the *tubu* knockdown was used as a marker for the sysRNAi-potency, 16 hours after injection of ds*tubu*. As control, locusts were injected with dsRNA for *gfp* and, 6 days later, they were injected with *tubu* dsRNA. A second control group that was treated twice with *gfp* dsRNA was also taken into account. Since in this group the *tubu* gene expression was not silenced, these locusts were used to determine the normal physiological *tubu* expression levels. Our results show that knocking down the *tubu* transcript level was significantly less potent when *vha16* was down regulated, *i.e.* in comparison to the first control group (Fig. 2A). Still, it remained possible that silencing *vha16* had some effect on the *tubu* transcript levels, rather than impairing the uptake of RNAi. Therefore, we also silenced *vha16* and measured the *tubu* transcript levels. The *tubu* expression levels remained indistinguishable upon silencing *vha16* from these in the *gfp* dsRNA injected control conditions (Fig. 2B). Second, we assessed whether silencing *clath* could also impair the sysRNAi-response. To ascertain that the observations were not specific for silencing *tubu*, we chose a different marker gene, namely *gapdh*, to determine the knockdown potency. The knockdown of *gapdh* was significantly less robust when

clath was down regulated (Fig. 2C), while silencing *clath* had no direct effect on the *gapdh* transcript levels (Fig. 2D).

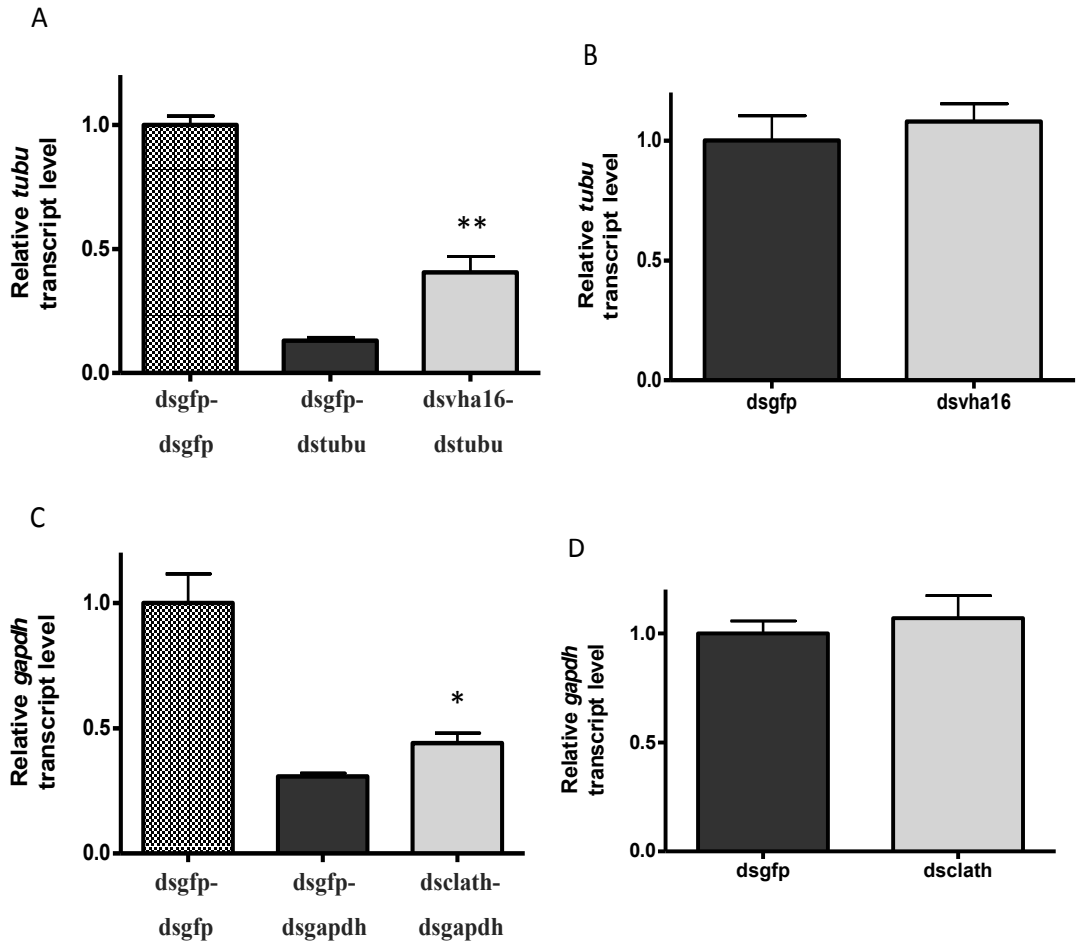


Fig. 2. *clath* and *vha16* are involved in mediating sysRNAi. (A) The locusts were first injected with 150 ng of ds*gfp* or ds*vha16* and 6 days later with 150 ng of *dsgfp* or *dstubu*. The different treatment groups are indicated as dsgfp-dsgfp, dsgfp-dstubu and dsvha16-dstubu. Sixteen hours after the second injection, the *tubu* transcript level in the midgut was determined by means of qPCR. Statistical analyses were performed between dsgfp-dstubu and dsvha16-dstubu (mean ± SEM; n = 8; **: $p < 0.005$). (B) The *tubu* transcript level was determined 6 days after injection of ds*gfp* or ds*tubu* (mean ± SEM; n = 6-8). (C) The locusts were first injected with 150 ng of ds*gfp* or ds*clath* and 4 days later injected with 150 ng of ds*gfp* or ds*gapdh*, indicated as dsgfp-dsgfp, dsgfp-dsgapdh and dsclath-dsgapdh, respectively. Statistical analyses were performed between dsgfp-dsgapdh and dsclath-dsgapdh (mean ± SEM; n = 8; *: $p < 0.05$). (D) The *gapdh* transcript level was determined four days after injection of ds*gfp* or ds*clath* (mean ± SEM; n = 6-7).

6.3.2. Scavenger receptors are important for sysRNAi

Since the scavenger receptors constitute a large group of proteins and in *Drosophila* it was shown that multiple SRs are involved in the uptake of dsRNA (Saleh *et al.*, 2006; Ulvila *et al.*, 2006), we used well-known inhibitors of the SR-family, namely Poly(I) and DS (Abrams *et al.*, 1992; Pearson *et al.*, 1993; Krieger and Herz, 1994; Pearson *et al.*, 1995; Whitman *et al.*, 2000). Regarding the fact that an adult desert locust possess, in average, 200 µl hemolymph, and that administration of 400 µg/ml of Poly(I) or DS to the medium of *Drosophila* L2 cells could inhibit > 85% of the SR-activity, 0.1 mg of the inhibitors was injected into the body cavity of desert locusts (which would correspond to 500 µg/ml in the hemolymph). One hour later, 150 ng of *tubu* dsRNA was injected and the potency of the *tubu* knockdown was compared to control groups that were injected with the same quantity of chemically related substances that do not bind to SRs, namely Poly(A) and CS (Abrams *et al.,* 1992; Pearson *et al.*, 1993). Our data demonstrate that blocking SRs significantly inhibited systemic silencing of *tubu* (Fig. 3A), while similar *tubu* expression levels were observed after injection of the inhibitors and the control compounds (Fig. 3B). In addition, we tested whether administration of lower Poly(I) quantities could also inhibit the sysRNAi-response in the desert locust. Therefore, we injected 5 µg of Poly(I) into the body cavity of the desert locust and one hour later these locusts were injected with 150 ng of ds*tubu*. The knockdown of *tubu* (16 hours p.i.) was significantly less robust in these locusts than in locusts that were first injected with Poly(A) and subsequently with ds*tubu* (Fig. 3C).

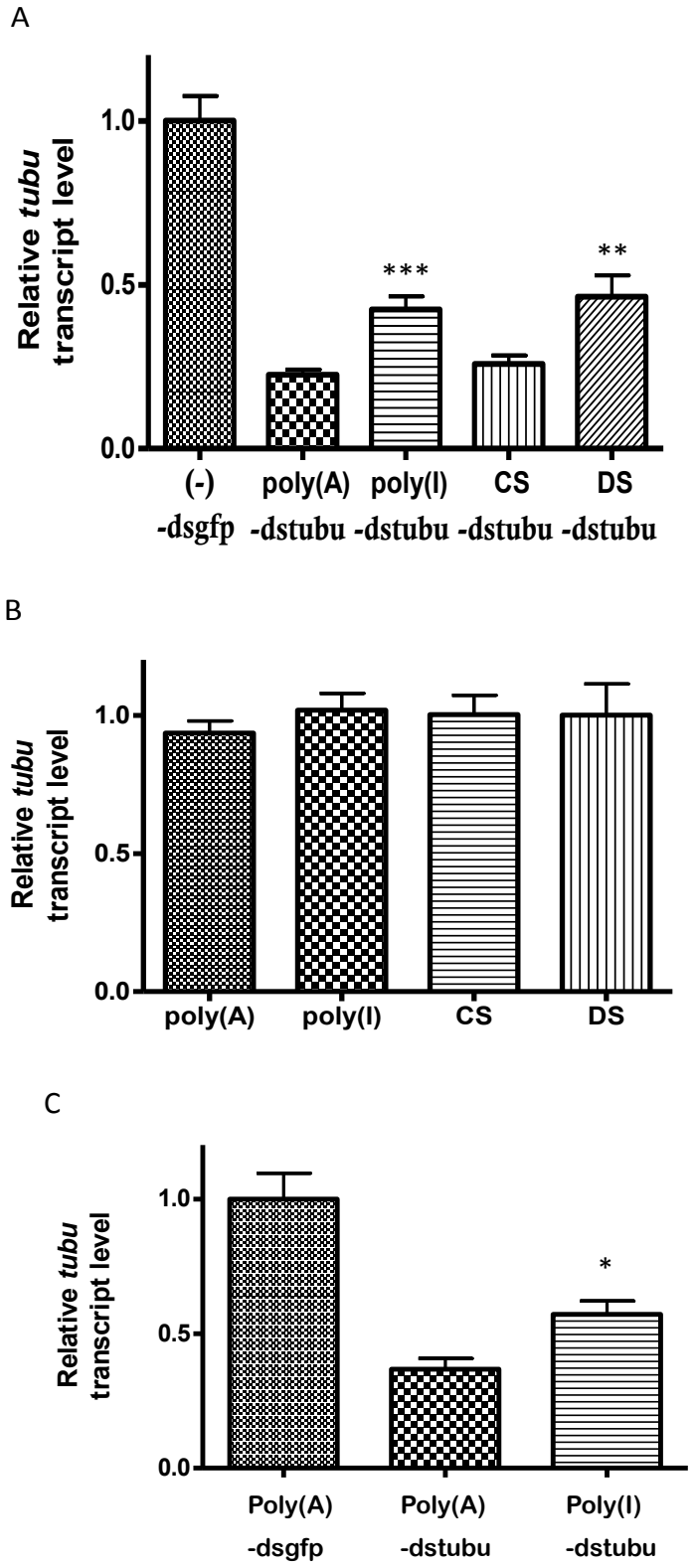


Fig. 3. Scavenger receptors facilitate sysRNAi. (A) Four days old adult locusts were first injected with 0.1 mg polyinosine (Poly(I)), polyadenosine (poly(A)), dextran sulphate (DS) or chondroitin sulphate (CS) and one hour later injected with 150 ng of dsRNA of *tubu*, indicated as Poly(I)-dstubu, Poly(A)-dstubu, DS-dstubu and CS-dstubu, respectively. A second control group that was once injected with 150 ng of ds*gfp*, indicated as (-)-dsgfp, was also accounted

for. Sixteen hours after the dsRNA injection, the *tubu* transcript level in the midgut was determined using qPCR. Statistical analyses were performed between the Poly(I)-dstubu and Poly(A)-dstubu, and between the DS-dstubu and CS-dstubu groups (mean ± SEM; n = 16-23; **: $p < 0.005$; ***: $p < 0.0001$). (B) Injection of poly(I) or DS, in comparison to injection of Poly(A) or CS, respectively, had no direct effect on the *tubu* expression level. The expression level was determined one hour after the injection (mean ± SEM; n = 6). (C) Four days old adult locusts were first injected with 5 µg Poly(A) or Poly(I), and (one hour later) injected with 150 ng ds*gfp* or ds*tubu*, designated as Poly(A)-dsgfp, Poly(A)-dstubu or Poly(I)-dstubu, respectively. The tubu transcript level was determined in the midgut by means of qPCR . Statistical analysis was performed between Poly(A)-dstubu and Poly(I)-dstubu (mean ± SEM; n = 8-10; *: $p < 0.05$).

6.3.3. Silencing the lipophorin receptor transcript had no detectable effect on the sysRNAi-response

In the *S. gregaria* transcriptome database, we found an *lpr* sequence that displays a widespread tissue distribution, yet with the highest expression level in the fat body of the desert locust (Fig. 4A). Next, we silenced this receptor by means of RNAi. However, injection of adult locusts with 150 ng of dsRNA only reduced the transcript level by 50% (data not shown). With the aim to enhance the knock down efficiency, 5th larval locusts were first injected with 300 ng of dsRNA and subsequently adult locusts were injected a second time (this approach was proven successful for increasing the knockdown efficiency of *apo1/2* and *apo3*, Chapter 5). Nonetheless, the knockdown efficiency remained relatively modest (Fig. 4B). Still, these locusts were subsequently injected with dsRNA of *gapdh* and, 16 hours later, we assessed the knockdown of *gapdh* with qPCR. Our experimental data were unable to confirm the involvement of *lpr* in RNAi (Fig. 4C).

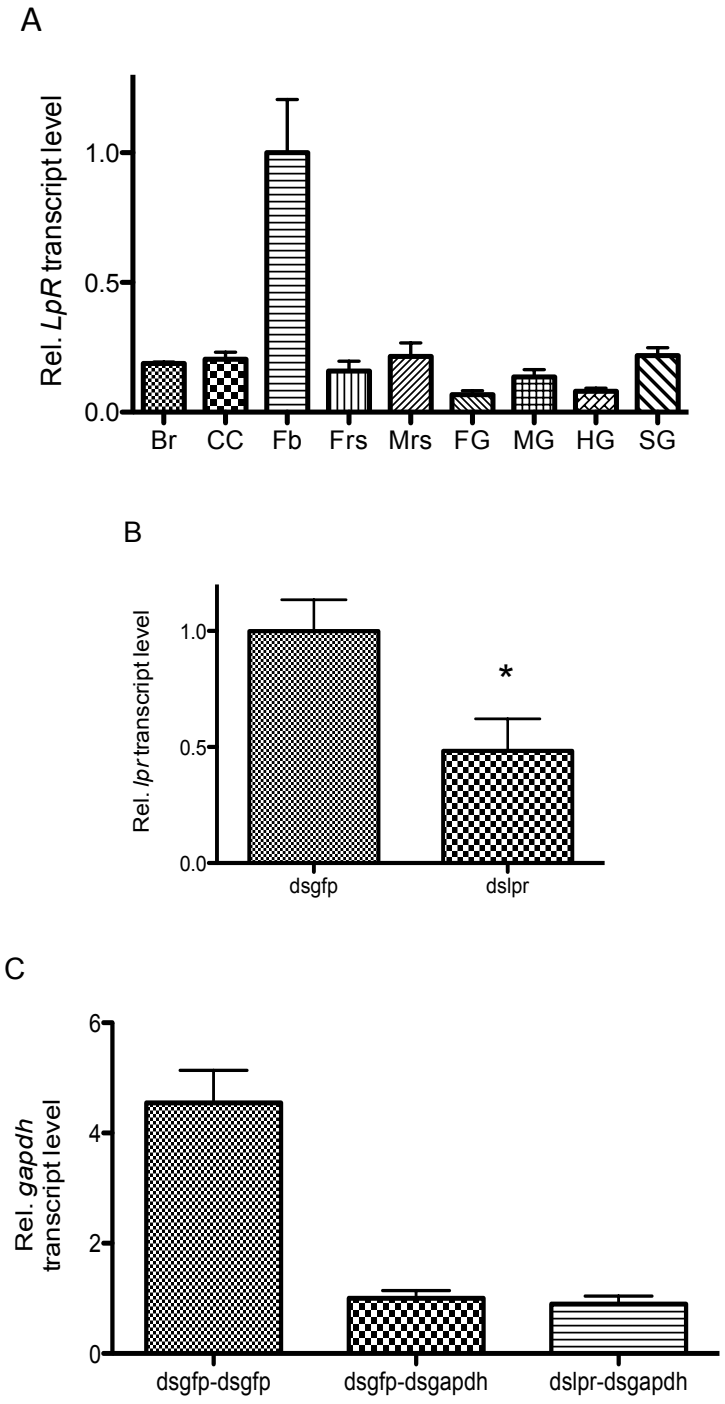
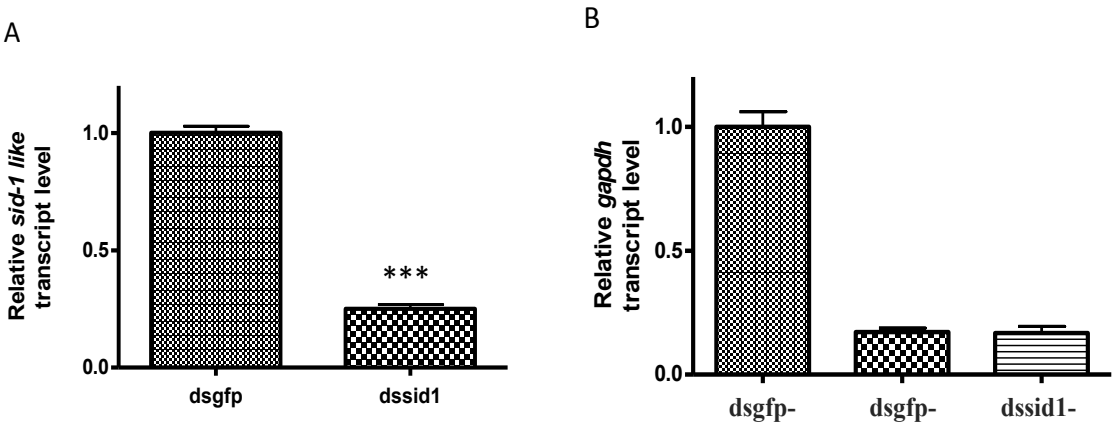


Fig. 4. The role of the *lpr* gene in sysRNAi remains inconclusive. (A) The tissue transcript profile for *lpr*. Each bar represents the mean of two independent pools of adult males (40 and 10 animals/pool) and three independent pools of adult females (40 and 10 animals/pool) (mean ± SEM) (Br= brain, TG= thoracic ganglia, SOG= suboesophageal ganglion, CC= corpora cardiaca, Frs= female reproductive system, Mrs= male reproductive system, Fb= fat body, FG= foregut, MG= midgut, HG= hindgut and SG= salivary glands). (B) Silencing of *lpr* was verified in the midgut using qPCR, 5th larval instar locusts were injected with 300 ng of *lpr* or ds*gfp*. In

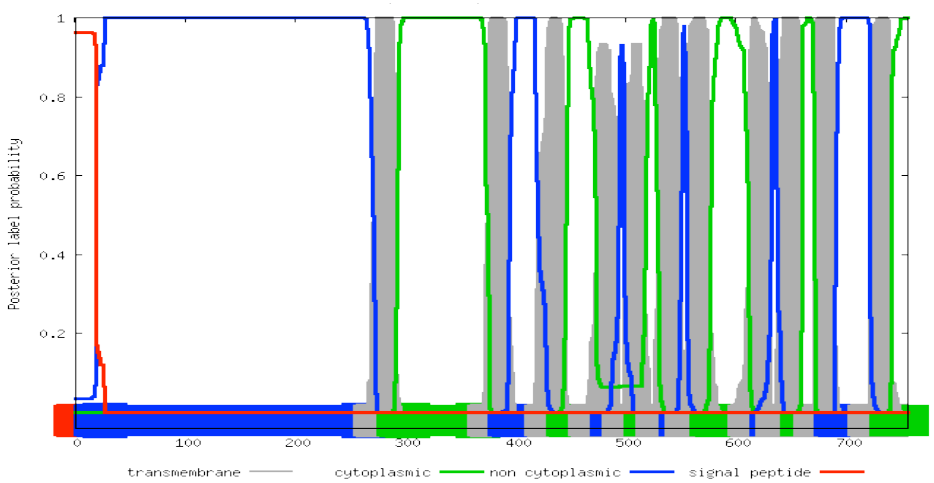
their adult stage, these locusts were treated for a second time with a dsRNA-injection and, six days later, the knockdown was assessed in the midgut by means of qPCR (means ± SEM, n = 8-9; * = $p < 0.05$). (C) Next, these locusts were also injected with 150 ng of dsRNA for *gapdh*. Sixteen hours later, the knockdown of *gapdh* was determined in the midgut by using qPCR. Locusts that were three times injected with ds*gfp* are indicated as dsgfp-dsgfp. Locusts that were twice injected with ds*gfp* or ds*lpr* and once with ds*gapdh* are indicated as dsgfp-dsgapdh, dslpr-dsgapdh (mean ± SEM; n = 10-11).

6.3.4. Silencing the *sid1 like* gene generate no clear effects on the RNAi-efficiency

Silencing *Sg-sid1 like* did not generate a clear effect on the RNAi-efficiency (Fig. 5B). Nonetheless, the knockdown of *sid1 like* was verified by means of qPCR (Fig. 5A). Moreover, sequence comparison of the deduced amino acid sequence of *Sg-sid1 like* showed higher sequence similarity with the Cholesterol Uptake Protein 1 (ChUP1, previously known as TAG-130) than with SID1 of *C. elegans*, which was also the case for all other insect *sid1 like* sequences investigated. *Ce*-ChUP-1 was also predicted to have a highly similar topological structure as the *Sg*-SID1 like protein (Fig. 4C) and comparison of their N-terminal extracellular domain revealed regions with high conservation with *Ce*-ChUP1, but not with *C*e-SID1 (Fig. 4D).



C



D

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sg-SID1         MMGRACCLVLSCVLVHLVQLLHGTEQQTEN--KNLSTIILNGNYSDYYTFNVNRT-VE--
ce-ChUP1        -MRTSQA-------IFILIFLDSV--------RNQSPQVIPAKWDVVYEKETGHNMSL--
ce-SID1         -----------MIRVYLIILMHLVIGLTQNNSTTPSPIITSSNS-SVLVFEISSKMKMIE

sg-SID1         --------YIFQFPASEDMIDKPA----RITVECKDSNRSFPVLVV-VRQQKGVLSWQLP
ce-ChUP1        --------TVFRFQVKE--QYSVA----RIIMSCNESTEHNPLLAV-FREKLAILSLQVP
ce-SID1         KKLEANTVHVLRLELDQSFILDLTKVAAEIVDSSKYSKEDGVILEVTVSNGRDSFLLKLP

sg-SID1         LLVETESDSLDYSRTSRTLCPDNSRYFAGNNVKTL--------------------YQYIIV
ce-ChUP1        LIVD----NYEYSQVARTLCPFTEY-KEGEAFTV---------------------------
ce-SID1         -----------------TVYPNLKLYTDGKLLNPLVEQDFGAHRKRHRIGDPHFHQNLIV

sg-SID1         SISTASNQNLSVSLLVS----RKEDFRIRLSDTRNITVL-----PAEPQYFAFRFPQSGP
ce-ChUP1        --EVTSSRPVHYNFRAE----LVQNFYLYNNSQRLVTAS-----ASEPVYLRYDIPGD-V
ce-SID1         TVQSRLNADIDYRLHVTHLDRAQYDFLKFKTGQTTKTLSNQKLTFVKPIGFFLNCSEQNI

sg-SID1         DTVLLRVDSDSQVCMMVSIQNMSCPVFDLE---QNVHFEGFWETVNVRGGITLP--RDAF
ce-ChUP1        DSVAVHLDSNSTICMTVSVQKIGCPVFDLP---DNVNSMGLHQTMTTSATIPVE--KSR-
ce-SID1         SQFHVTLYSEDDICANLITVPANESIYDRSVISDKTHNRRV-LSFTKRADIFFTETEISM

sg-SID1         PLGFYVVFVVKGDDVDCSGPD-----HPIIKP-RNKTLSFSLSPSITYQKYLV----AAC
ce-ChUP1        MSSFYVVFVVNTNDDLCSEILSIKPNKPTKFPLRMKSFNVTIESSMKIFDYTIPIVFWAC
ce-SID1         FKSFRIFVFIAPDDSGCSTNTSRKSFNEKK------------------------------

sg-SID1         V----------------------
ce-ChUP1        ILLLVTIVVFVYHYFDGIWERR
ce-SID1         ----------------------
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Fig. 5. (A) Silencing of *sid1 like* was verified in the midgut using qPCR, 6 days after injection of 150 ng *sid1 like* dsRNA (mean ± SEM; n = 8-10; ***: p < 0.0001). (B) Locusts were injected with 150 ng *gfp* or *sid1 like* dsRNA and subsequently with 150 ng ds*gfp* or ds*gapdh*, indicated as dsgfp-dsgfp, dsgfp-dsgapdh or dssid1-dsgapdh, respectively. The *gapdh* knockdown was determined 16 hours later with qPCR (n = 9-10). (C) The predicted topological structure of *Ce*-ChUP1 using Phobius software. (D) Sequence comparison of the N-terminal extracellular domain of *Sg*-SID1 like, *Ce*-ChUP1 and *Ce*-SID1 using the Clustal Omega Sequence Alignment software (EMBL-EBI). Regions with high similarity between *Ce*-ChUP1 and *Sg*-SID1 like are underlined in blue.

6.4. Discussion

By silencing two distinct components of the clathrin-dependent endocytosis machinery and by pharmacological inhibition of SRs, we have demonstrated that SR-mediated uptake contributes to the robust RNAi-response in the desert locust, *S. gregaria.* In contrast, knocking down *Sg-sid1 like* generated no clear effects on the RNAi-efficiency. Moreover, sequence comparison revealed higher sequence similarity with *Ce*-ChUP1 than with *Ce*-SID1. Studies have demonstrated that *Ce*-ChUP1 is not involved in dsRNA-uptake in *C. elegans* (Tomoyasu *et al.*, 2008), but mediates the uptake of dietary cholesterol (Valdes *et al.*, 2012). Whether *Sg-sid1 like* is also involved in cholesterol transport rather than dsRNA-uptake remains to be determined.

Although *S. gregaria* and *C. elegans* display a robust sysRNAi-response, these data suggest that *S. gregaria* uses different dsRNA-uptake mechanisms. On the other hand, it might seem surprising that S2 cells, an sysRNAi-sensitive cell line derived from the largely sysRNAi-insensitive *D. melanogaster,* use the same dsRNA-entry route (Saleh *et al.*, 2006; Ulvila *et al.*, 2006). However, the tissue specificity and expression levels of components involved in this uptake-mechanism may determine the difference in success of the dsRNA-transport. Next, as pointed out by several recent reports, the efficiency of the cell-autonomous RNAi-machinery may also be a determinate factor for the potency of the knockdown (Swevers *et al.*, 2011; Garbutt and Reynolds, 2012). The observation that the sysRNAi-response was only partially inhibited may still suggest the presence of alternative parallel dsRNA-uptake mechanisms. Nevertheless, we have previously demonstrated that injection of pg quantities of dsRNA per mg tissue can already evoke a clearly detectable RNAi knockdown in *S. gregaria* (Chapter 3). Therefore, one can expect that residual cell entry of dsRNA, due to incomplete silencing or inhibition of the uptake mechanisms, would still result in activation of the RNAi-response. This might indeed have been the case in our experiments, keeping in mind the technical limitations of the applied procedures. First, competitively blocking the SRs with poly(I) or DS may have been reversible and incomplete. Second, a knockdown (established using dsRNA-injections in the desert locust) does not generate *loss-of-function* effects to the same degree as a complete knockout. Therefore, to date it remains uncertain whether additional parallel cell entry routes are also functional in the desert locust.

Besides the ability of nematode cells to efficiently take up dsRNA from their extracellular environment via SID1, studies have demonstrated that the RNAi-signal is also amplified by RNA-dependent-RNA-Polymerases (RdRPs) in these cells (Smardon *et al.*, 2000; Sijen *et al.*, 2001), ensuring high potency and prolonged effectiveness of RNAi. Whereas ticks also posses RdRPs, they seem to have lost *sid1* related sequences (Kurscheid *et al.*, 2009). Aung *et al.* (2011) demonstrated that the hard tick, *Haemaphysalis longicornis*, uses SRs to mediate sysRNAi. Yet, regarding the importance of RdRPs in systemic gene silencing in plants, fungi and nematodes (Cogoni and Macino, 1999; Mourrain *et al.*, 2000; Sijen *et al.*, 2001), RdRPs may also attribute to systemic RNAi in ticks. In contrast, knowing that insects lack *RdRP-like* sequences in their genomes (at least for these insects whose genome has been sequenced), it might seem surprising that insects can display robust sysRNAi-responses. One possible explanation could reside in the presence of a highly robust cell-autonomous RNAi-machinery. This is supported by the high sensitivity of the RNAi-response in the *S. gregari*a (Chapter 3), *T. castaneum* (Tomoyasu and Denell, 2004) and *L. migratoria* (Luo *et al.*, 2012), illustrating that only small doses of dsRNA are needed to activate the core RNAi-machinery in these insects.

In humans, dsRNA entry is also facilitated by SRs, while it was suggested that long dsRNA induces interferon responses, rather then being an RNAi-signal (Limmon *et al.*, 2008; Dieudonne *et al.*, 2012). Taken together, this implies conservation of SR-mediated dsRNA-uptake. Furthermore, several studies have highlighted the importance of endocytosis-based spreading of the RNAi-signal in the nematode, *C. elegans* (Hinas *et al.*, 2012; Jose *et al.*, 2012). While direct participation of SR-mediated endocytosis in dsRNA-uptake in the desert locust remains to be demonstrated, a role in dsRNA-uptake might not seem surprising, knowing that SR-mediated endocytosis is a well-described uptake route for polyanionic ligands (Platt and Gordon, 1998) and dsRNA-uptake via this uptake mechanism has been directly demonstrated in mammalian cell lines (to activate the interferon response; Dieudonne *et al.*, 2012; Limmon *et al.*, 2008) and *Drosophila* S2 cells (Saleh *et al.*, 2006; Ulvila *et al.*, 2006).

Moreover, since (i) SRs are well-known receptors for lipophorins, (ii) their vertebrate homologues, lipoproteins, can promote the *in vivo* delivery of DNA (Guevara *et al.*, 1999; Guevara *et al.*, 2010), and (iii) *Sg*-lipophorins bind to dsRNA in the hemolymph

(Chapter 5), a role for the lipophorins in the cellular delivery of dsRNA might not seem illogical. Whether the entire lipophorin-dsRNA complex is taken up or whether the lipophorins promote the delivery of dsRNA to dsRNA-transporters remains undetermined. Although we have also investigated the involvement of LpR in RNAi, the knockdown efficiency was probably insufficient to draw final conclusions. Further research is needed to clarify the mechanisms of dsRNA-uptake in insects.

In conclusion, we demonstrated that SR-mediated endocytosis contributes to sysRNAi in the desert locust. Since this study is the first report on an *in vivo* dsRNA-transport mechanism in an sysRNAi-sensitive insect species, it contributes to a better understanding of the mechanisms of sysRNAi in insects and can possibly support the search for the improvement of the sysRNAi-efficiency in other species. In addition, since SRs are well-known receptors of lipophorins, this study may further support a possible role of lipophorins in the cellular delivery of RNAi.

Chapter 7

General discussion

Chapter 7: General discussion

7.1. Delivery of dsRNA to induce RNAi in *S. gregaria*

In the previous chapters, the results obtained in the context of my PhD-project were described. By analysing different aspects of the systemic (sys)RNAi-response, we identified key role mechanisms that might influence the success of RNAi in the desert locust (summarized in Fig. 1).

By testing different dsRNA-delivery methods, we were able to demonstrate that injection of dsRNA into the locust's body cavity is a highly efficient method to induce RNAi-effects, while on the other hand the RNAi-response is less responsive towards orally delivered dsRNA. Moreover, although high dsRNA-doses were used, RNAi upon oral delivery of dsRNA remained unsuccessful (Chapter 3). In line with this, dsRNA was rapidly degraded in the gut lumen by the action of dsRNases, a group of nucleases that were exclusively found in insects and crustaceans (Chapter 5). Therefore, it seems logical to assume that the dsRNases may help to prevent the onset of RNAi-responses upon orally delivered dsRNA. Nevertheless, knocking down vital genes (via injection of dsRNA), such as *tubu* (Chapter 3) or *clath* (Chapter 6), resulted in locust mortality. Thus, developing dsRNA-delivery methods that could promote RNAi-responses for orally delivered dsRNA might be of particular interest for pest management programs for locusts.

In addition, it was fascinating to see that dsRNA is also rapidly degraded in the hemolymph (Chapter 5). At state, the exact nature of the dsRNA-degrading enzymes in the hemolymph remains undetermined. Nevertheless, the dsRNA-degradation was markedly less efficient in the serum than in the midgut juice (Chapter 5). In this respect, the efficiency of the dsRNA-degradation might help to determine the difference in success upon intra-abdominal and oral injection of dsRNA.

Furthermore, the majority of the residual dsRNA in the hemolymph seemed to be bound by lipid-protein complexes, termed lipophorins, which typically act in energy transport in insects (Chapter 5). Where several recent reports illustrated that lipophorins also function as circulating scavengers for bacterial and fungal

pathogens (Gotz *et al.*, 1997; Dettloff *et al.*, 2001; Whitten *et al.*, 2004; Cheon *et al.*, 2006; Ma *et al.*, 2006), our study brings in that they can also recognize viral associated molecular patterns, in particular dsRNA-fragments. This is in accordance to reports made on vertebrate lipoproteins that can recognize several viral particles (Singh *et al.*, 1999). Nevertheless, the exact role of the dsRNA-binding activity of lipophorins remains unclear. Under our experimental conditions, lipophorins did not seem to protect dsRNA against degradation (Chapter 5). Yet, in order to draw final conclusions, this issue should be investigated in more detail. On the other hand, since the vertebrate homologues, lipoproteins, were shown to mediate *in vivo* cellular delivery of DNA (Guevara *et al.*, 2010), it is plausible to consider that lipophorins could also promote cellular delivery of dsRNA in insects. This hypothesis was further supported by the fact that scavenger receptors, which are well-known receptors for lipophorins, were found to participate in the sysRNAi-response of *S. gregaria* (Chapter 6).

Although potent dsRNA-degradation activity was observed in the serum, this shouldn't necessarily affect the success of RNAi. Moreover, keeping in mind the fact that dsRNA is an important virus-associated molecular pattern that could represent the genome of a dsRNA-virus, control over the entire dsRNA-titer might be of particular importance. Yet, to avoid a conflict of interest (for instance for energy-transport or recognition of other pathogen-associated molecular patterns by lipophorins), locusts shouldn't sustain higher dsRNA-levels than needed to saturate the antiviral RNAi-response. Therefore, a tightly regulated dsRNA-sensing system that degrades the excess of dsRNA and guides the residual dsRNA to the cells (possibly via lipophorins) might be of interest. However, this would require the presence of a highly sensitive RNAi-response, which was supported by the fact that injection of pg quantities of dsRNA per mg tissue induced potent gene silencing effects in the desert locust (Chapter 3). A possible explanation for the high sensitivity of the RNAi-response might reside in the presence of a highly sensitive cell-autonomous RNAi-machinery or (unidentified) RdRP-independent dsRNA-amplification mechanisms.

In Chapter 6, we demonstrated that scavenger receptors (SRs) and clathrin-dependent endocytosis are involved in the sysRNAi-response. Nonetheless, we were unable to demonstrate the direct involvement of SR-mediated endocytosis

in the cellular uptake of dsRNA. Yet, since it was previously shown that *Drosophila* and vertebrate cells utilize SR-mediated endocytosis to import dsRNA (Saleh *et al.*, 2006; Ulvila *et al.*, 2006; Limmon *et al.*, 2008; Dieudonne *et al.*, 2012), a role in *in vivo* dsRNA-uptake in *S. gregaria* might seem plausible. However, the RNAi-machinery is located in the cytoplasm. Thus, the dsRNA should be redirected from the general endocytosis pathway to the cytoplasm. Herein, lysosomal acidification by the action of Vha16 might be of particular importance. In this respect, Saleh *et al.* (2006) demonstrated that silencing *vha16* in *Drosophila* S2 cells impairs the delivery of dsRNA to the cytoplasm, while the initial dsRNA-uptake into endocytotic vesicles remained intact. In addition, we have shown that knocking down *vha16* attenuated the *in vivo* RNAi-response in *S. gregaria* (Chapter 6). Once the dsRNA reached the cytoplasm, *Sg*-Dcr2 and *Sg*-Ago2 will participate in RNAi (as demonstrated in Chapter 4). Finally, our results suggest that reduced expression levels of *Sg-dcr2* and *Sg-ago2* might contribute to the more moderate RNAi-responses in the adult reproductive systems of the desert locust (Chapter 4).

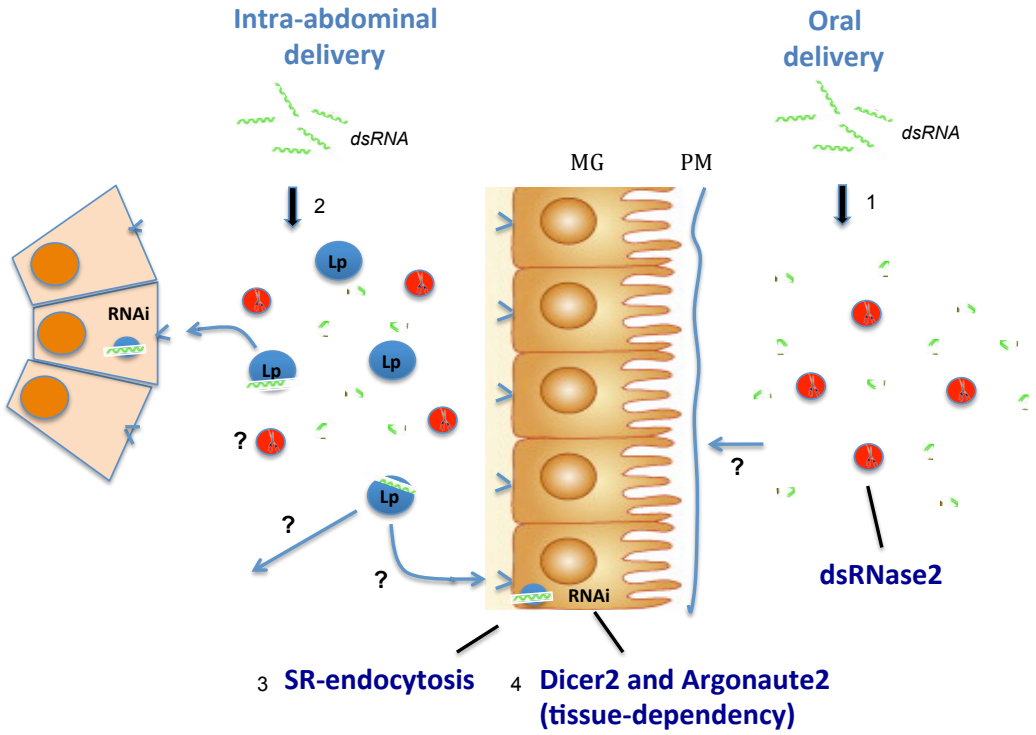

Fig. 1. The proposed model for the mechanisms of sysRNAi in the desert locust. (1) Oral delivery of dsRNA (depicted in green) results in the rapid degradation of the dsRNA by the action of dsRNases, with in particular an important role for dsRNase2. Yet, whether the desert locust possesses the necessary dsRNA-transport mechanisms to take up dsRNA from the gut lumen remains undetermined. (2) Injection of dsRNA into the locust's body cavity also results in rapid degradation. Still, a small part of the dsRNA remains intact and will be bound by lipophorins (Lp). Currently, the identity of the dsRNA-degrading enzymes in the hemolymph remains undetermined. In addition, the exact role of Lp in signalling to the immune response also remains unclear. Nevertheless, a role in the delivery of dsRNA might not seem surprising, since (3) SR-mediated endocytosis (SR-endocytosis) contributes to the uptake of dsRNA. (4) Once the dsRNA has been delivered to the cytoplasm of the target cell, it will be further processed by Dicer2 and Argonaute2 and induce gene silencing (MG= midgut; PI= peritrophic membrane).

7.2. Applied techniques

While the results presented in this PhD-thesis bring in new fundamental insights into the mechanisms of sysRNAi in insects, several techniques were also optimized. First, our data have significantly improved RNAi-based reverse genetic research on locusts. Prior to the onset of this PhD-project, several micrograms of

dsRNA were used for RNAi-experiments on locusts. Yet, our data propose that a single injection of much lower dsRNA-quantities is sufficient to saturate the RNAi-pathway, thereby reducing the cost of RNAi-experiments by the lab. Moreover, we demonstrated that most tissues investigated in our lab could be targeted with RNAi. To obtain potent gene silencing in the adult reproductive tissues, we illustrated that a longer time span is required. Therefore, in order to obtain a potent knockdown in these tissues, it might be of interest to start the dsRNA-injections in the 5th larval stage.

In addition, during this PhD-thesis, we have demonstrated that the 'RNAi on RNAi' approach is a highly effective method to identify genes involved in the RNAi-process. Therefore, we selected two RNAi-sensitive marker genes to assess the efficiency of RNAi, namely *tubu* and *gapdh*. In addition, preliminary results demonstrated that 16 hours after the dsRNA injection, the knockdown efficiency is relatively potent, yet still not complete, making it an ideal time point to assess differences in the RNAi-efficiency. Although many different tissues could probably be used, the midgut tissue was proven a suitable site to investigate the RNAi-potency. Furthermore, we recommend adding an additional control where the direct impact on the physiological expression level of the marker gene is assessed.

We have also developed an *ex-vivo* assay to assess the dsRNA-integrity in body fluids that was based on visualizing the intact dsRNA by means of electrophoresis. In addition, we have successfully applied phenol-chloroform extraction to dissociate nucleoprotein complexes and so improved our understanding on the dsRNA-binding proteins in the serum.

Finally, a method to purify nucleic acids-binding proteins from an agarose gel was developed. In particular, this led to the identification of dsRNA-binding proteins.

7.3. Future prospects

7.3.1. Oral delivery of lipid- or polymer-dsRNA complexes

In general, mammals are insensitive towards *in vivo* injected siRNAs, mainly due to enzymatic degradation in the blood and inefficient cellular uptake. However, multiple studies have shown that injection of siRNA:lipid particles, such as

liposomes and lipoplexes, can help to overcome these obstacles and can induce successful knockdown effects in mammals (summarized in Shim and Kwon, 2010). Moreover, feeding *D. melanogaster* larvae with dsRNA-fragments encapsulated in liposomes conferred these flies sensitivity towards orally delivered RNAi (Whyard *et al.*, 2009).

Successful *in vivo* siRNA-delivery to mammalian tissues has also been obtained using siRNA complexed with polyethylenimine (PEI), biodegradable cationic polysaccharides (e.g. chitosan) or cationic polypeptides (e.g. atelocollagen, poly(L-lysine) and protamine), summarized by Shim and Kwon (2010).

Regarding the potential of RNAi-based insect pest management, dsRNA-lipid and dsRNA-polymer complexes could be tested for their ability to generate gene silencing effects upon oral delivery to locusts.

Finally, oral delivery of bacteria that secrete dsRNA-fragments has also been successful to induce gene silencing effects in insects (Tian *et al.*, 2009), and might also serve as a possible approach.

7.3.2. Role of individual scavenger receptors in RNAi

We obtained positive evidence for the involvement of SRs in the RNAi-pathway of the desert locust (Chapter 6). However, to serve this purpose, we used general inhibitors of the SR-family. In the *S. gregaria* transcriptome database, we found five class B SR transcripts and one class C SR transcript. A further issue to be considered is the identification of the individual SRs involved in the uptake of dsRNA. Therefore, by means of *in vivo* 'RNAi on RNAi' and via transfection of cell lines that have low dsRNA-uptake efficiency (*e.g.* Chinese Hamster Ovary (CHO) cells) with individual *Sg*-SRs, the contributing SRs could be identified. For the latter, the uptake of Cy3-labelled dsRNA could be compared to this of cells that are transfected with an empty vector. Finally, administering lipophorins to the cell medium has potential to shed light on the possible role of lipophorins in the delivery of dsRNA.

7.3.3. Transfection of insect cell lines with expression constructs for RNAi-components of *S. gregaria* and *C. elegans*

With the aim of improving gene silencing in organisms with a weak (sys)RNAi-response, including many lepidopterans and dipterans (Belles, 2010), *Drosophila* cell lines can be transfected with RNAi-components known to mediate key steps of the (sys)RNAi response in the desert locust or in *C. elegans*. These cells can subsequently be analysed for RNAi-efficiency. Regarding the improvement of the cell-autonomous RNAi machinery, the cells could be transfected with *Sg-ago2* and *Sg-dcr2*. RdRPs were identified to play an important role in the robust RNAi machinery of the nematode *C. elegans*, but no orthologs were found in insects (Huvenne and Smagghe, 2010). In this context, cells can be transfected with the *C. elegans* RdRPs *EGO*-1 (Smardon *et al.*, 2000) and *RRF*-1 (Sijen *et al.*, 2001). To improve the systemic spread of the RNAi-signal, the cells can be transfected with *Sg-SRs* or *Ce-sid1*. In addition, transfection of these cells with *dsRNAses* could further elucidate their role in the RNAi-pathway.

7.3.4. Antiviral role of RNAi

Regarding the antiviral role of sysRNAi, it is plausible to consider the fact that a more robust (sys)RNAi-response might result in more potent antiviral immune responses. To study this matter we propose to test whether improvement of the RNAi-response of *Drosophila* cells (§ 7.3.3.) could result in enhanced antiviral immunity. This research could bring new fundamental insights in how the potency of antiviral immunity might differ between different species, and can lead to new methods to combat viral infections in vector and beneficial insects. Herein, the impact on acute as well as latent viral infections could be assessed.

In addition, several viruses can evade the RNAi-response through mechanisms, such as the production of viral encoded suppressors of RNAi (VSRs), and *Drosophila* S2 cells are persistently infected with multiple viruses (including the flock house virus (FHV) that encodes a VSR) (Flynt *et al.*, 2009). Therefore, it might be interesting to assess whether the presence of these latent viral infections affect the RNAi-efficiency of the S2 cells. In this context, by targeting specific regions of the viral genomes with dsRNA-fragments, combined with

physical selection, based on qPCR, of cells with reduced viral presence, FHV-free S2 cells can be selected and their RNAi-potency can be assessed.

Moreover, the desert locust could be *in vivo* injected with viruses that encode VSRs, such as the FHV or the Cricket Paralyzing Virus (CPV). In this way, we could determine whether the presence of viruses might affect the (sys)RNAi potency in the desert locust.

Furthermore, based on the data delivered in this thesis, we could identify RNAi key steps in the antiviral immunity of the desert locust. Therefore, the sysRNAi-response can be impaired by *in vivo* injection of pharmacological inhibitors or dsRNA-fragments directed against RNAi-components. Next, by measuring the viral presence with qPCR, we could identify their role in antiviral immunity

7.3.5. Gene specific knockdown efficiency

The type of gene can also influence the efficiency of RNAi. For instance, it has become increasingly clear that efficient silencing of receptors can be challenging, a group of proteins with typically low abundance (unpublished data from our research group).

Keeping in mind the fact that RNAi generates knockdown effects, the residual transcript levels might not be detected by RISC. In this context, the transcript detection limit for RISC would be in the same range for all genes. Thus, the efficiency of the knockdown would be directly correlated to the physiological expression level of the target gene. In this context, we propose to investigate the correlation between the RNAi-efficiency and the absolute transcript level of the mRNA target. For this purpose, one could compare the knockdown efficiency for different genes with high and low expression levels. The absolute transcript levels could be measured with digital PCR.

Moreover, viruses persistently present in insects might survive by remaining below this RNAi-detection limit. In regard of this hypothesis, the residual target transcript levels following RNAi and the viral RNA-levels would be expected to be in the same range. On the other hand, VSRs could directly affect the RNAi-detection limit, allowing higher quantities of viral transcripts in the cell.

7.3.6. miRNA-directed regulation of gene expression

Whereas this thesis focuses on siRNA-directed RNAi, miRNAs are considered to regulate several major physiological processes in insects (Lucas and Raikhel, 2013). In the *S. gregaria* transcriptome database, we found sequence information for *dicer-1* and *drosha*, two essential enzymes in the miRNA-biogenesis. Silencing their expression by using RNAi, and therefore impairing miRNA-directed gene regulation, could result in severe physiological defects, including locust development, ecdysis, spermatogenesis, oogenesis, reproduction and phase transition. This approach has been used to demonstrate the importance of the miRNA-biogenesis in regulation of oocyte development in *B. germanica* (Tanaka and Piulachs, 2012), and of metamorphosis in *L. migratory* (Wang *et al.*, 2013) and in *B. germanica* (Gomez-Orte and Belles, 2009).

In order to construct a small RNA library for the desert locust, we could purify small RNAs and determine their sequences by means of RNA sequencing. Next, differential expression of miRNAs between different conditions and *in vivo* experiments designed to block particular miRNAs could be used to identify regulatory miRNAs in particular physiological processes.

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